DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP-HPLC

Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai.

In partial fulfillment for the award of the Degree of

MASTER IN PHARMACY
(Pharmaceutical Analysis)

APRIL-2012

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPATTI ROAD,

COIMBATORE -641048.

DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP-HPLC

Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai.

In partial fulfillment for the award of the Degree of

MASTER IN PHARMACY

(Pharmaceutical Analysis)

APRIL-2012

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPATTI ROAD,

COIMBATORE -641048.

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,

Principal,

K.M.C.H. College of Pharmacy,

Kovai Estate, Kalapatti Road,

Coimbatore -641048. (T.N)

CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP- HPLC"** *is a bonafide research work carried out by Mr.* Rambabu Cherukuri ***(Reg. No: 26107226)***, *Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, under the guidance of* ***Prof.*** **Dr. A. Rajasekaran**, *Dept of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy during the academic year 2011-2012.*

Date:

Place: Coimbatore.

Signature,

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,
Principal

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,

Principal,

Department of Pharmaceutical Analysis,

K.M.C.H. College of Pharmacy,

Coimbatore -641048.

CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP- HPLC"** *is a bonafide research work carried out by Mr.* Rambabu Cherukuri ***(Reg. No: 26107226),*** *Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, under my supervision and guidance during the academic year 2011-2012.*

I am fully satisfied with his performance and work with great pleasure. I forward this Dissertation work for evaluation.

Date:

Place: Coimbatore.

Signature,

Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,
Guide

DECLARATION

I am here by stating that, to the best of my knowledge and belief, the project report entitled **"DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP- HPLC"** *being submitted for the partial fulfillment of Master of Pharmacy in Pharmaceutical Analysis for the academic year 2011-2012 of KMCH. College of Pharmacy affiliated to The Tamilnadu Dr. M.G.R. Medical University carried out under the guidance of* **Prof. Dr. A. Rajasekaran, M. Pharm, Ph.D.,** *at the Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore.*

I abide that all the data presented in this report will be treated with utmost confidentiality.

Signature,

Date: **Rambabu Cherukuri**

Place: Coimbatore.

EVALUATION CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP- HPLC"** *submitted by Mr.* Rambabu Cherukuri ***(Reg. No: 26107226),*** *to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of* ***Master of Pharmacy****, in* ***Pharmaceutical Analysis****, is a bonafide research work carried out by the candidate at K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, the same was evaluated by us during academic year 2011-2012.*

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

ACKNOWLEDGEMENT

My dissertation entitled **"DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF RELATED SUBSTANCE IN IRINOTECAN HCl FORMULATION AND ITS STABILITY INDICATING STUDIES BY RP- HPLC"** *would not have been a feasible one without the grace of god almighty who gave me morale till the completion of my project.*

To begin with I would like to thank ***Dr. Nalla G. Palanisamy****, Chairman, and* ***Dr. Thavamani D. Palanisamy****, Managing Trustee, K.M.C.H. College of Pharmacy, Coimbatore for all the facilities, which have been provided to us at the institution, enabling me to do work of this magnitude.*

I always remain indebted to my Academic Guide ***Dr. A. Rajasekaran****,* ***M. Pharm****,* ***Ph.D.,*** *Principal, K.M.C.H College of Pharmacy for his constant insight, guidance, countless serenity, encouragement and painstaking efforts in my project work. I am indebted to his kindness and never failing co – operation and provided facilities.*

My esteemed and beloved staff ***Mr. J. Dharuman, M. Pharm.,*** *Professor, Department of Pharmaceutical Analysis, KMCH College of Pharmacy, for their sensible help and suggestions.*

I owe my heartful thanks to my esteemed and beloved staff ***Mr. I. Ponnilavarasan, M. Pharm.,*** *Assist. Professor, Department of Pharmaceutical Analysis,* ***Mr. K. Suresh Kumar, M. Pharm, Ph.D.,*** *Assist. Professor, Dept. Pharm. Analysis,* ***Mr. G. Arihara Sivakumar, M. Pharm.,*** *Professor, Dept. Pharmacology,* ***Mr. K.T. Manisenthil Kumar, M. Pharm.,*** *Professor, Dept. Pharmacology,* ***Mr. S. Ramachandran, M. Pharm.,*** *Assist. Professor, Dept. Pharmacology,* ***Dr. K.S.G. Arul Kumaran,*** *Professor, Dept. Pharmaceutics,*

Mr. N. Arun Kumar, M. Pharm., *Professor, Dept. Pharmaceutics,* ***Mr. C. Sankar M. pharm, Ph.D.,*** *Professor, Dept. Pharmaceutics,* ***Mr. K.K. Sivakumar, M. Pharm.,*** *Assist. Professor, Dept. Pharm. Chemistry,* ***Mrs. S. Hurmath Unnissa, M. Pharm.,*** *Assist. Professor, Dept. Pharm. Chemistry,* ***Mr. C. Sundaramoorthi, M. Pharm.,*** *Professor, Dept. Pharm. Biotechnology and* ***Mr. R.*** **Arivukkarasu,** ***M. Pharm.,*** *Assist. Professor, Dept. Pharmacognasy, for their sensible help and suggestions.*

My special thanks to all teaching and non-teaching staff members of KMCH College Pharmacy, Coimbatore, Library and computer lab faculties who directly or indirectly gave a helping hand during the course of study.

I express my sincere thanks to all my classmates, senior, juniors, and friends for their timely help and co-operation.

Last but not the least; I thank all those persons who directly or indirectly helped me in this project work to finish with great success.

Yours sincerely,
Rambabu cherukuri

ABBREVATIONS

HPLC	High Performance Liquid Chromatography
UV	Ultra violet
IP	Indian Pharmacopoeia
USP	United States Pharmacopoeia
M.W	Molecular weight
e.g.	Example
%	Percentage
PDA	Photo Diode Array
ACN	Acetonitrile
MET	Methanol
mg	Milligram
g	gram
µg	Microgram
mL	Milliliter
w/w	Weight by weight
µg/mL	Microgram per milliliter
°C	Degree Celsius
t	Time
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
M.P	Melting Point

RS	Related substance
CPT-11	Irinotecan HCl
SN-38	7-ethyl 10-hydroxy camptothecin
min^{-1}	Per one minute
mL^{-1}	Per one milliliter

INDEX

ABSTRACT

A simple isocratic RP-HPLC stability indicating method has been developed and subsequently validated for the determination of Irinotecan HCl and its related substance (SN-38) in pharmaceutical dosage forms as per ICH guidelines. The separation achieved on a reversed phase Phenomenex Luna C_{18} Column, 5µ, 250 × 4.60 mm as a stationary phase and 0.5% trichloro acetic acid: Acetonitrile: Methanol (60: 20: 20 v/v/v) as mobile phase at a flow rate of 1.0 mL/min. The UV detection was performed at 372 nm. The retention time (R_t) for Irinotecan HCl and SN-38 was found to be 8.65 and 7.30 min respectively. The detector response was linear in the concentration range of 30-150 µg/mL. The respective linear regression equation being Y= 5233.x + 13299 with R^2 0.999. The percentage of Irinotecan HCl in pharmaceutical dosage form was found to be 100.5% and the percentage of related substance (SN-38) in formulation was found to be 0.19%. The limit of detection of Irinotecan was 0.014 µg/mL and the limit of quantification was 0.045 µg/mL. The results of the study showed that, the proposed RP-HPLC method was simple, rapid, precise, accurate and stability indicated, which can be used for the routine determination of Irinotecan HCl and its related substance (SN-38) in pharmaceutical dosage form.

KEY WORDS: Irinotecan HCl, SN-38, RP-HPLC, Related substance.

CHAPTER -1

INTRODUCTION

Chapter 1

INTRODUCTION [1-24]

IMPURITY STUDIES [1-15]

Impurities in a drug substance (a new chemical entity of therapeutic interest) or drug product (a drug substance formulated into a suitable product for administration to patient) may cause serious side effects and hence we need to isolate and characterize impurities.

The following definition of impurity is currently under consideration by the regulatory bodies, which is likely to be included in the future guidance:

Impurity: any entity of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Related substances: These substances are structurally related to the drug substance and may be identified or unidentified degradation products or impurities arising from a manufacturing process or during storage of a material.

Impurity profile has become essential as per various regulatory requirements. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's). Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity; thus, revealing the need and scope of impurity profiling of drugs in pharmaceutical research The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has also published guidelines for validation of methods for analyzing impurities in new drug substances, products, residual solvents and microbiological impurities.[2-5]

As per ICH guidelines Impurities are listed alphabetically as given below.

- By-product
- Degradation product

- Interaction product
- Intermediate
- Penultimate intermediate
- Related product
- Transformation product

By-products: The unplanned compounds generated in the reaction to produce API are generally called by-products. For example during the synthesis of paracetamol we get diacetylated paracetamol which is the unexpected product called by-product.

Degradation products: The compounds produced as a result of decomposition of the material of interest or API is often called degradation products. Due to degradation, impurities may also be formed. Degradation of beta lactum ring in penicillin and cephalosporin are the examples of degradation products. 2-(4-formylphenyl) propionic acid, 2-(4-methylphenyl) propionic acid , 2-(4-ethylphenyl) propionic acid , 4- isobutylacetophenone , 2-(4-n-propylphenyl) propionic acid and 2-(4-n-butylphenyl) propionic acid are the reported degradation products of ibuprofen.

Interaction products: This term is slightly more inclusive and more difficult to evaluate than the two previously described, i.e., by-products and degradation products, in that it takes into account interactions that could possibly occur between various involved chemicals – purposely or inadvertently.

Intermediates: The planned compounds produced during synthesis of the desired substance are called intermediates, especially if they have been isolated and characterized.

Penultimate intermediate: As the name implies, this is the last compound in the synthetic chain just preceding the production of the ultimate desired compound.

Related products: As suggested previously, the term “related products” tends to imply that the impurity is similar to the drug substance, and it thus tends to downplay the negativity frequently attached to the term “Impurity”.

Transformation products: This is relatively a term that relates to theorized and non-theorized products that may be produced in the reaction.

Table 1. Description of impurity types and their sources

	Impurity type	Impurity source
1	Process-related drug substance	- Organic - Starting material - Intermediate - By-product - Impurity in starting material
2	Process-related drug product	- Organic or inorganic - Reagents, catalysts, etc
3	Degradation drug substance or drug product	- Organic - Degradation products
4	Degradation drug product	- Organic - Excipient interaction

1.1.Classification of impurities

According to ICH guidelines, impurities can be broadly classified in to three categories for the drug substance produced by chemical synthesis:

- Organic impurities (starting materials, process-related products, inter- mediators, and degradation products).
- Inorganic impurities (salts, catalysts, ligands, and heavy metals or other residual metals).
- Residual solvents (organic and inorganic liquids used during production and/or recrystallization).

1.2. Need to Isolate and Characterize Impurities

Impurities are generally assumed to be inferior to API because they might not have the same level of pharmacologic activity. However, they are not necessarily always inferior. From the standpoint of its usage, the drug substance is compromised in terms of purity even if it contains another material with superior pharmacologic or toxicologic properties. At first pass this may not

be readily apparent; however, on further thought it will become clear that if we are to ensure that the accurate amount of the drug substance is being administered to the patient, we must assess its purity independent of the extraneous materials. Therefore, any extraneous material present in the drug substance or active ingredient must be considered an impurity even if it is totally inert or has superior pharmacologic properties, so that an appropriate evaluation of its content in the drug product can be made. The control of low-level impurities is of great importance when a drug is taken in large quantities. Impurities can also affect the purity of API or can be harmful to patients.

1.2. Sources of Impurities

Impurities can originate from various sources, the most obvious source of impurities is from synthesis of API, where intermediates and by-products may be carried into the API as impurities or become a source of other impurities resulting from them. Following are the sources of the impurities

1.3.1. Crystallization- related impurities

Polymorphism is the term used to denote crystal systems where a substance can exist in different crystal packing arrangements, all of which have the same elemental composition. It is also possible to have crystal systems where the substance exists in different crystal packing arrangements, each of which has a different elemental composition; this phenomenon is known as solvatomorphism. The nature of the crystal structure of a given material can influence the following properties:

- Conductivity
- Crystal hardness
- Crystal shape and color
- Density
- Diffusivity
- Dissolution rate
- Electrolytic conductivity

- Enthalpy of transitions
- Heat capacity
- Heat of solution
- Hygroscopicity
- Latent heat of fusion
- Melting or sublimation properties
- Phase diagrams
- Rates of reactions
- Refractive index
- Solubility
- Surface tension
- Viscosity
- Volume

1.3.2. Stereochemistry-related impurities

Substances having similar chemical structure but having different spatial orientation are considered as stereo-chemical impurities in the API. Stereoisomerism is possible in molecules that have any of the following characteristics:

- One or more center of chirality
- Helicity (e.g., helical nature of tertiary structure of proteins, polysaccharides and nucleotides)
- Planar chirality(e.g., polycyclophanes)
- Axial chirality (e.g,.spiranes with cyclic skeleton)
- Torsional chirality (e.g., torsion about double or single bonds like cis and trans isomers and rotomers)
- Topological asymmetry (e.g., catenanes).

Pthalidomide tragedy is the good example for stereo-chemical impurities.

1.3.3 Residual solvents

Water is commonly present in drug products. As a result, water is by far the most commonly found volatile impurity in drug products, and most of the time it is not even considered an impurity. A number of solvents that are used for the synthesis of the API or formulation of the drug product can be present in the drug product. The content of these solvents, which are commonly called organic volatile impurities (OVI), is generally determined by the OVI methods specified in the compendia.

1.3.4. Synthetic intermediates and by-products

In addition to the residual solvents, polymorphic, solvatomorphic, and chiral impurities mentioned previously, impurities in a pharmaceutical compound or a new chemical entity (NCE) can originate during the synthetic process from raw materials, intermediates, and/or by-products. The solvents used in synthesis are also likely to involve a number of impurities that may extend from trace levels to critical quantities that can react with various chemicals used in the synthesis, to give rise to other impurities.

1.3.5. Formulation-related impurities

As mentioned before, many impurities in a drug product can originate from excipient used to formulate a drug substance. In addition, a drug substance is subjected to a variety of conditions in the process of formulation that can cause its degradation or have other undesirable reactions. For example if heat is used for drying purpose or for some other reason, it can hasten degradation.

1.3.6. Impurities arising during storage

A number of impurities can originate during storage (shelf life) or shipment of drug products. It is essential to carry out stability studies to predict, evaluate, and ensure drug product safety. Stability, however, can have different meanings to different people, based on their discipline in the pharmaceutical sciences and industry.

1.3.7. Method related impurity

Terminal sterilization by autoclave for the diclofenac injectables known to produse impurity of 1-(2, 6-dichlorophenyl) indolin-2-one. Intramolecular cyclic reaction of diclofenac sodium cause the formation of indolinone impurity during autoclave method.

1.3.8. Mutual interaction amongst ingredients

In multi component dosage forms there may be a chance to get mutual interaction, particularly this occurs in multivitamin dosage forms. the presence of nicotinamide in a formulation containing four vitamins (nicotinamide, pyridoxine, riboflavin, and thiamine) can cause the degradation of thiamine to a sub-standard level within a one year shelf life of vitamin B-complex injections.

1.3.9 Functional group related degradations

API containing ester groups can readily be hydrolysed causing the formation of degraded products. For Eg aspirin, benzocaine, benzylpenicillin, oxazepam and lincomycin,cefotaxime, ethyl paraben, and cefpodoxime undergo hydrolysis readily causing the degradation of API.

API containing hydroxyl group directly bonded to an aromatic ring (*viz* phenol derivatives such as catecholamines and morphine), conjugated dienes (*viz* vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (especially flavorings) are all susceptible to oxidative degradation.

Some of the API undergoes degradation on exposure to light. API like Ergometrine [27], nifedipine [28], nitroprusside, riboflavin and phenothiazines are veryliable to photo-oxidation.

1.4. ANALYTICAL METHOD DEVELOPMENT

Analytical method development for the quantification of low-level impurities present in pharmaceuticals can be thought of as a three-step process:

1. Sample set selection for analytical method development

2. Screening of chromatographic conditions and phases, typically using the linear-solvent-strength model of gradient elution
3. Optimization of the method to fine-tune parameters related to ruggedness and robustness. This can be accomplished using a factorial optimization approach.

1.5 CHARACTERISATION OF IMPURITIES

1.5.1. SPECTROSCOPIC AND SEPARATION METHODS

The following spectroscopic measurement techniques have been used for characterizing and isolating impurities; most of these are very useful as detectors for chromatographic methods:

- Ultraviolet(UV)
- Infrared(IR)
- Raman spectroscopy
- Mass spectrometry(MS)
- Nuclear magnetic resonance(NMR)
- Capillary electrophoresis (CE)
- Thin-layer chromatography(TLC)
- Gas chromatography (GC)
- High- pressure liquid chromatography (HPLC)
- Supercritical fluid chromatography (SFC)
- Chiral separations

Ultraviolet spectrophotometry (UV) at a single wavelength furnishes minimum selectivity of analysis; however, with the current accessibility of diode array detectors, it is conceivable to obtain sufficient simultaneous information at various wavelengths to assure greater reliability.

Infrared spectrophotometry (IR) affords specific information on some functional groups that offer selectivity and aloe quantification.

Raman spectroscopy is based on the measurement of scattered electromagnetic radiation resulting from the irradiation of matter.

Thin-layer chromatography coupled with densitometric detection is a highly sensitive method for quick assessment of the purity of various compounds including reference standards.

Mass spectrometry (MS) provides excellent structural information, and, based on the resolution of the instrument, it may be an effective tool for differentiating molecules with small differences in molecular weight. However, it has finite uses as a quantitative procedure.

Nuclear magnetic resonance spectroscopy (NMR) provides reasonably detailed structural information on a molecule and is an extremely useful method for characterization of impurities; however, its use as a quantitative method is limited.

Capillary electrophoresis is an effective technique in situations where very low quantities of samples are available and high resolution is essential.

Gas chromatography is an extremely useful technique for quantification. It can afford the desired resolution, selectivity and ease of quantification.

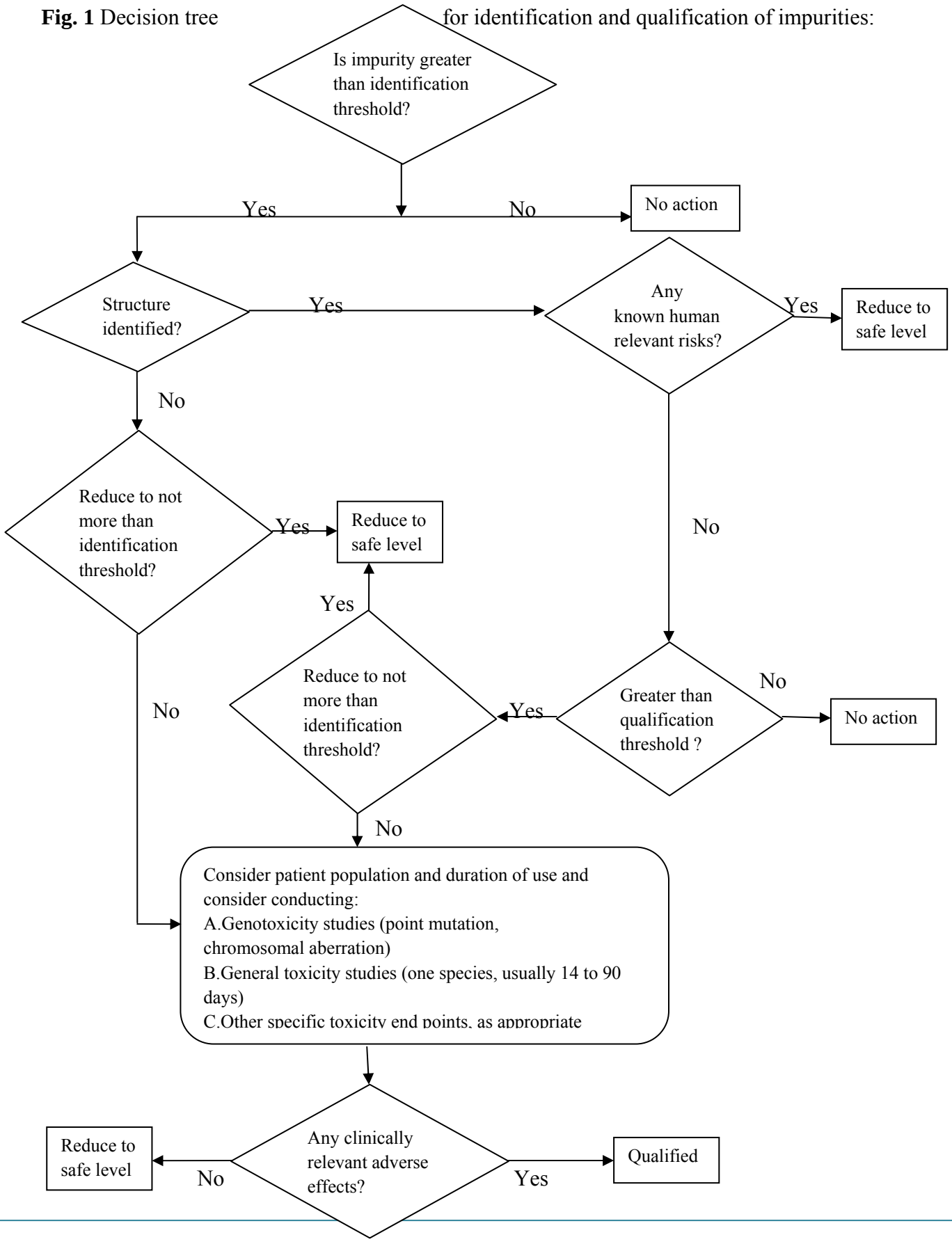
High-pressure liquid chromatography is often referred to as high-performance liquid chromatography today. Both terms can be abbreviated as HPLC, and the terms are used interchangeably by chromatographers. The applications of this very effective technique have been significantly expanded for the pharmaceutical chemist by the use of a variety of detectors such as fluorescence, electrometric, MS and so forth.

Supercritical fluid chromatography (SFC) offers some of the advantages of GC in terms of detection and of HPLC in terms of separations, in that volatility of the sample is not of paramount importance.

1.6. REGULATORY GUIDANCE ON IMPURITIES

The United States Food and Drug Administration (FDA) have endorsed the guidance prepared under the auspices of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Fig. 1 Decision tree for identification and qualification of impurities:



2. METHOD DEVELOPMENT [16-19]

Numerous methods are required to characterize drug substances and drug products. Specifications may include description; identification; assay (of composite sample); tests for organic synthetic process impurities, inorganic impurities, degradation products, residual solvents, and container extractables; tests of various physicochemical properties, chiral purity, water content, content uniformity, and antioxidant and antimicrobial preservative content; microbial tests; dissolution/disintegration tests; hardness/friability tests; and tests for particle size and polymorphic form. Some of these tests may be precluded, or additional tests may be added as dictated by the chemistry of the pharmaceutical or the dosage form. Due to the variability in specific tests required to fully characterize a pharmaceutical, it is difficult to provide a comprehensive discussion to address all aspects of pharmaceutical development. However, the requisite tests can be broadly subdivided into three main categories:

- Tests concerned with solid-state characterization
- Compendial tests
- Quantitative tests to characterize drug substance and drug product composition

Quantitative tests to characterize drug substance and drug product composition require that significant consideration be given to method development. Methods such as thin layer chromatography, gas chromatography, HPLC, supercritical fluid chromatography, and capillary electrophoresis are used for pharmaceutical analysis. High-pressure, or high-performance, liquid chromatography (both are abbreviated as HPLC or simply LC; is predominantly used in the pharmaceutical industry for evaluations of a large variety of samples. It is the method of choice for checking purity of new chemical entities, monitoring changes in synthetic procedures or scale up, evaluating new formulations, and carrying out quality control/assurance of the final drug product. As mentioned before, phase-appropriate HPLC method development must be given serious consideration during method development.

2.1 HPLC METHOD DEVELOPMENT STEPS:

The steps will be discussed in the same order as they would be investigated during the method development process. The rational will be illustrated by focusing on developing a stability-indicating HPLC-UV method for related substances (impurities). The principles, however, will be applicable to most other HPLC methods.

The following is a suggested method development timeline for a typical HPLC-UV related substance method. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually. Each of these steps will be discussed in more detail in the following paragraphs.

Step 1: **Define method objectives and understand the chemistry of analytes**

Determination of the goals for method development (e.g., what is the intended use of the method?), and understand the chemistry of the analytes and the drug product.

Step 2: **HPLC Initial conditions**

Development of preliminary HPLC conditions to achieve minimally acceptable separations. These HPLC conditions will be used for all subsequent method development experiments.

Step 3: **Procedure for Sample preparation**

Develop the suitable sample preparation scheme for the drug product

Step 4: **Standardization**

Determination of the appropriate standardization method and the use of relative response factors in calculations.

Step 5: **Final method optimization or robustness**

Identification of the "weaknesses" of the method and optimization of the method through experimental design and understand the method performance with different conditions, different instrument set ups and different samples.

Step 6: **Validation of method**

The complete method validation according to ICH guidelines

2.1.1. DEFINE METHOD OBJECTIVES

There is no absolute end to the method development process. The question is what is the "acceptable method performance"? The acceptable method performance is determined by the objectives set in this step. This is one of the most important considerations often overlooked by scientists. In this section, the different end points (i.e., expectations) will be discussed in descending order of significance.

2.1.1.1. Analytes:

For a related substance method, determining the "significant and relevant" related substances is very critical. With limited experience with the drug product, a good way to determine the significant related substances is to look at the degradation products observed during stress testing. Significant degradation products observed during stress testing should be investigated in the method development.

Based on the current ICH guidelines on specifications, the related substances method for active pharmaceutical ingredients (API) should focus on both the API degradation products and synthetic impurities, while the same method for drug products should focus only on the degradation products. In general practice, unless there are any special toxicology concerns, related substances below the limit of quantitation (LOQ) should not be reported and therefore should not be investigated.

In this stage, relevant related substances should be separated into 2 groups:

2.1.1.2. Significant related substances: Linearity, accuracy and response factors should be established for the significant related substances during the method validation. To limit the workload during method development, usually 3 or less significant related substances should be selected in a method.

2.1.1.3. Other related substances: These are potential degradation products that are not significant in amount. The developed HPLC conditions only need to provide good resolution for these related substances to show that they do not exist in significant levels.

2.1.2. Resolution (Rs)

A stability indicating method must resolve all significant degradation products from each other. Typically the minimum requirement for baseline resolution is 1.5. This limit is valid only for 2 Gaussian-shape peaks of equal size. In actual method development, Rs = 2.0 should be used as a minimum to account for day to day variability, non-ideal peak shapes and differences in peak sizes.

2.1.3. Limit of Quantitation (LOQ)

The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be 0.05% or less to ensure the results are accurate up to one decimal place. However, it is of little value to develop a method with an LOQ much below this level in standard practice because when the method is too sensitive, method precision and accuracy are compromised.

2.1.4. Precision, Accuracy

Expectations for precision and accuracy should be determined on a case by case basis. For a typical related substance method, the RSD of 6 replicates should be less than 10%. Accuracy should be within 70 % to 130% of theory at the LOQ level.

2.1.5. Analysis time

A run time of about 5-10 minutes per injection is sufficient in most routine related substance analyses. Unless the method is intended to support a high-volume assay, shortening the run time further is not recommended as it may compromise the method performance in other aspects (e.g., specificity, precision and accuracy.)

2.1.6. Adaptability for Automation

For methods that are likely to be used in a high sample volume application, it is very important for the method to be "automatable". The manual sample preparation procedure should be easy to perform. This will ensure the sample preparation can be automated in common sample preparation workstations.

2.1.7. UNDERSTAND THE CHEMISTRY

Similar to any other research project, a comprehensive literature search of the chemical and physical properties of the analytes (and other structurally related compounds) is essential to ensure the success of the project.

2.1.8. Chemical Properties

Most sample preparations involve the use of organic-aqueous and acid-base extraction techniques. Therefore it is very helpful to understand the solubility and pKa of the analytes. Solubility in different organic or aqueous solvents determines the best composition of the sample solvent. p^{Ka} determines the pH in which the analyte will exist as a neutral or ionic species. This information will facilitate an efficient sample extraction scheme and determine the optimum pH in mobile phase to achieve good separations.

2.1.9. Potential Degradation Products

Subjecting the API or drug product to common stress conditions provides insight into the stability of the analytes under different conditions. The common stress conditions include acidic pH, basic pH, neutral pH, different temperature and humidity conditions, oxidation, reduction and photo-degradation. These studies help to determine the significant related substances to be

used in method development, and to determine the sample solvent that gives the best sample solution stability.

In addition, the structures of the analytes will indicate the potential active sites for degradation. Knowledge from basic organic chemistry will help to predict the reactivity of the functional groups. For example, some excipients are known to contain trace level of peroxide impurities. If the analyte is susceptible to oxidation, these peroxide impurities could possibly produce significant degradation products.

2.1.10. Sample Matrix

Physical (e.g., solubility) and chemical (e.g., UV activity, stability, pH effect) properties of the sample matrix will help to design an appropriate sample preparation scheme. For example, Hydroxypropyl Methylcellulose (HPMC) is known to absorb water to form a very viscous solution, therefore it is essential to use mostly organic solvents in sample preparation.

2.2. INITIAL METHOD CONDITIONS

The objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments. A common mistake is that scientists spend too much time at this stage trying to get a perfect separation.

2.2.1. Preliminary HPLC Conditions

In order to develop preliminary HPLC conditions in a timely fashion, scientists should use artificial mixtures of active pharmaceutical ingredients and related substances at relatively high concentrations (e.g., 1-2% of related substance relative to API) to develop the preliminary HPLC conditions. The concentration ratio between API and the related substances should be maintained to ensure the chromatography represents that of a real sample. Alternatively, a highly stressed sample (e.g., 5% degradation) can also be used at this stage. With the known composition and high levels of degradation products in the sample, one can evaluate the chromatography to determine whether there are adequate separations for all analytes. The high concentrations of related substances are used to ensure all peaks will be detected.

Computer assisted method development can be very helpful in developing the preliminary HPLC conditions quickly. Since the objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments, scientists should focus on the separation of the significant related substances instead of trying to achieve good resolution for all related substances. These significant related substances should be baseline resolved from each other with Rs > 2.0. After the preliminary method development, the HPLC conditions can be further fine-tuned at a later stage (see section 8, method optimization/ robustness) to achieve the required specificity for the other related substances.

2.2.3. Aged HPLC Column

An aged HPLC column should be used to develop the initial HPLC conditions. Usually it is more difficult to achieve the required resolution with an aged column (e.g., column with about 200 injections). This will reflect the worst case scenario likely to be encountered in actual method uses, and help the long-term method robustness.

In general, develop all methods with HPLC columns from the same vendor. The preferred brand of HPLC column should be selected primarily based on the long term stability and lot to lot reproducibility.

2.4. SAMPLE PREPARATION

2.4.1 Selection of Sample Solvent

This stage focuses on the selection of the sample solvent (for extraction) and the proper sample preparation procedures. Investigate the effect of sample solvents of different % organic, pH, extraction volume and extraction procedure on accuracy, precision, sensitivity (LOQ) and the changes in the chromatography (e.g., peak shape, resolution). Whenever possible use the mobile phase in the sample preparation. This will ensure that there will not be any compatibility issues between the sample solution and the HPLC conditions.

2.4.1.1. Accuracy: To investigate the accuracy in sample preparation (i.e., extraction efficiency), prepare a spiked solution by adding known amounts of related substances into a sample matrix. Compare responses of the spike solutions and the neat standard solutions to assess the recovery from the sample preparation. In this stage, since only one particular step is being investigated (i.e., sample preparation), close to theoretical recovery should be observed at this point (e.g., 90-110%).

2.4.1.2. Precision: Use the stressed sample to represent the worst case scenario and perform replicate sample preparations from the same sample composite. Investigate the consistency of the related substance profile (i.e., any missing peaks?) and the repeatability results from these preparations.

Another objective is to determine the sample concentration that gives an acceptable LOQ (Signal to Noise ratio, S/N) in low level spike concentrations. The sample concentration should be low enough to maintain linearity and precision, but high enough to achieve the desired LOQ. For example, if the ICH reporting limit for this drug product is 0.1%, the LOQ of the method should be less than 0.05% (i.e., desired LOQ, in %). By using spike sample solutions of very diluted concentrations for the significant related substances, estimate the concentrations that give a S/N of about 10 for the significant related substances. This estimated concentration is the approximate LOQ concentration (i.e., estimated LOQ concentration, in µg/mL).

The following equation can be used to estimate the target sample concentration for the method:

Target sample concentration = estimated LOQ concentration (µg/mL) x 1/desired LOQ (%) x 100%

2.5. STANDARDIZATION

2.5.1. Area % method

If the response of the active pharmaceutical ingredient is linear from LOQ to the nominal sample concentration, use the % area approach where the related substance is reported as % area. This is the most straightforward approach, and doesn't require the preparation of standard solutions. It also has the highest precision since preparation to preparation variation will not affect the results. However, in order to ensure the concentration is linear within this range, the sample

concentration is usually limited and this will reduce the method sensitivity (i.e., increase LOQ). In general, use this approach as long as the desired LOQ can be achieved.

2.5.2. External Standard method

Use the external standard method if the response of the active pharmaceutical ingredient is not linear throughout the whole range, or the desired LOQ cannot be achieved by the area % method. The concentration of standard solution should be high enough to ensure the standard solution can be prepared accurately and precisely on a routine basis, it should be low enough to approximate the concentration of related substance in the sample solution. In general, the standard concentration should correspond to about 5 % of related substances.

2.5.3. Wavelength Selection and Relative Response Factor

Generate the linearity plot of API and related substances at different wavelengths. At this point, Photodiode Array Detector can be used to investigate the linearity of the active pharmaceutical ingredient and related substances in the proposed concentration range. By comparing the linearity slopes of the active pharmaceutical ingredient and the related substances, one can estimate the relative response factors of the related substances at different wavelengths.

Disregard of whether Area % or External Standard approach is used, if the relative response factors of some significant related substances are far from unity, a response factor correction must be applied. The optimum wavelength of detection is the wavelength that gives the highest sensitivity (λ_{max}) for the significant related substances and minimizes the difference in response factors between those of the active pharmaceutical ingredient and the related substances. After the optimum wavelength is determined, use a highly stressed sample (e.g., 5% degradation) to verify that the selected wavelength will give the highest % related substance results.

2.5.4. Overall accuracy

A final check of the method performance is to determine the overall accuracy of the method. Unlike the accuracy from sample preparation, which simply compares the response of the analyte with and without spiking with matrix, the overall accuracy compares the % related substances calculated from an accurate solution with that of the theoretical value. The accuracy solutions are the solutions spiked with known concentrations of related substances and matrix. Since the

extraction efficiency, choice of wavelength and the bias in standardization influence the calculated related substance result, this is the best way to investigate the accuracy of the method. Overall accuracy reflects the true accuracy of the method.

2.6. METHOD OPTIMIZATION/ ROBUSTNESS

After the individual components of the method are optimized, perform the final optimization of the method to improve the accuracy, precision and LOQ. Use an experimental design approach to determine the experimental factors that have significant impact on the method. This is very important in determining what factors need to be investigated in the robustness testing during the method validation. To streamLine the method optimization process, use Plackett Burmann Design (or similar approach) to simultaneously determine the main effects of many experimental factors.

Some of the typical experimental factors that need to be investigated are:

HPLC conditions: % organic, pH, flow rate, temperature, wavelength, column age.

Sample preparation: % organic, pH, shaking/sonication, sample size, sample age.

Calculation/standardization: integration, wavelength, standard concentration, response factor correction.

Typical responses that need to be investigated are:

Results: precision (%RSD), % related substance of significant related substances, total related substances.

Chromatography: resolution, tailing factor, separation of all related substances.

2.7. METHOD VALIDATION

2.7.1. Robustness

Method validation should be treated as a "final verification" of the method performance and should not be used as part of the method development. Some of the typical method validation parameters should be studied thoroughly in the previous steps. In some cases, robustness can be completed in the final method optimization before method validation. At this point, the robustness experiments should be limited only to the most significant factors (usually less than 4 factors). In addition, unlike the final method optimization, the experimental factors should be

varied within a narrow range to reflect normal day to day variation. During the method validation, the purpose is to demonstrate that the method performance will not be significantly impacted by slight variations of the method conditions.

2.7.2. Linearity, Accuracy, Response Factor

Linearity, accuracy and response factors should be established for the significant related substances during the method validation. In order to limit the workload of method development, usually less than 3 significant related substances should be selected in a method. Therefore, the other related substances should not be included in these experiments.

2.7.3. System suitability criteria

It is advisable to run system suitability tests in these robustness experiments. During the robustness testing of the method validation, critical method parameters such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range. Consequently, the limits for system suitability tests can be estimated from these experiments.

3. DEGRADATION STUDIES [20-24]

A degradation product is defined as a chemical change in the drug molecule brought about over time and/or by action of, e.g., light, temperature, pH, or water, or by reaction with an excipient and/or the immediate container/closure system (also called decomposition product).

ICH Guideline on Stability Testing

Stress testing helps determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used. Stress testing is conducted to provide data on forced decomposition products and decomposition mechanisms. The severe conditions that may be encountered during distribution can be covered by stress testing. These studies should

establish the inherent stability characteristics of the molecule, such as the degradation pathways, and lead to identification of the degradation products and hence support the suitability of the proposed analytical procedures. The detailed nature of the studies will depend on the individual drug substance and type of drug product.

The 1987 Food and Drug Administration (FDA) Stability Guideline defines stability-indicating methodology as:

Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.

3.1.Drug Substance Degradation Studies:

Purposeful degradation studies of the drug substance include appropriate solution and solid-state stress conditions (e.g., acid/base hydrolysis, heat, humidity, oxidation, and light exposure, in accordance with ICH guidelines). Guidelines from the United States Pharmacopoeia (USP), ICH, and FDA provide a brief outline of drug substance conditions. The ICH guidelines specifically state

Stress testing is likely to be carried out on a single batch of material and to include the effect of temperatures in 10 ◦C increments above the accelerated temperature test condition (e.g., 50 ◦C, 60 ◦C, etc.); humidity where appropriate (e.g. 75% RH or greater); oxidation and photolysis on the drug substance plus its susceptibility to hydrolysis across a wide range of pH values when in solution or suspension.

3.1.1. Acid/Base Stress Testing:

Acid/base stress testing is performed to force the degradation of a drug substance to its primary degradation products by exposure to acidic and basic conditions over time. Functional groups

likely to introduce acid/base hydrolysis are amides (lactams), esters (lactones), carbamates, imides, imines, alcohols (epimerization for chiral centers), and aryl amines.

To initiate acid/base studies, a preliminary solubility screen of the drug substance is performed. Solubility of at least 1 mg/mL in 1 N acidic and basic condition is recommended for the acid/base stress testing; however, concentrations less than 1 mg/mL can be used if solubility is an issue. In some cases, a co-solvent may be necessary to achieve the target concentration. Special attention should be given to the drug substance structure when choosing an appropriate co-solvent.

Table 2. Guidance on Acid/Base Experimental Setup:

Samples:	Drug substance + acid (1 N HCl) Drug substance + base (1 N NaOH) Drug substance "as is" Acid control (1 N HCl) Base control (1 N NaOH)
Kinetic points:	0 – 1week

3.1.2. Thermal and Thermal/Humidity Stress Testing:

The goal of thermal and thermal/humidity studies is to force the degradation of drug substances over time to determine the primary thermal and/or humidity degradation products.

Table 3. Guidance for Thermal Stress Length of Study:

Temperature	Length of storage
30˚C	1 year
40˚C	6 months
50˚C	12 weeks
60˚C	6 weeks
70˚C	3 weeks
80˚C	11 days

Table 4. Guidance for Thermal/Humidity Experimental Setup:

Samples:	70˚C/30% RH **(**ambient humidity**)** 70˚C/30% RH
Time points:	0 – 6 week

3.1.3. Oxidation:

Oxidative studies are executed to force the degradation of drug substances to determine the primary oxidative degradation products. Oxidative degradation is a serious stability problem and can cause a major halt in pharmaceutical development. The 1987 Stability Guidelines state that a high oxygen atmosphere should be evaluated in stability studies on solutions or suspensions of the bulk drug substance.

Table 5. Guidance for Oxidative Degradation Experimental Setup:

Samples:	Oxygen with initiator Oxygen without initiator Argon with initiator Thermal control Initiator and antioxidant without drug substance
Time points:	0 – 10 days

3.1.4. Photostability:

The goal of the photostability studies is to force the degradation of drug substances via UV and fluorescent conditions over time to determine the primary degradation products. UV and visible light are the most energetic electromagnetic radiation sources to which pharmaceutical drug substances and drug products are typically exposed. A molecule absorbs light when an absorption band exists that overlaps to some extent with the incident light energy and a valence

electron in the relevant chromophore is raised to an excited state. Light stress conditions can also induce photo oxidation by free radical mechanisms.

Table 6. Guidance for Ultraviolet Degradation Experimental Set Up:

Samples:	Ultraviolet Thermal foil-wrapped control
Time points:	5× and 10 × ICH*

3.1.4.1.Ultraviolet Exposure:

ICH guidelines specify an exposure of 200 watt h/m2 for ultraviolet light confirmatory testing (1× ICH). Recommended stress conditions and time points to be tested are 5× and 10× ICH for solid drug substances

All samples should be taken at the appropriate kinetic time points and protected from any further light exposure. Light measurements should be taken in watts/m2 to be consistent with the ICH guidelines.

3.1.4.2.Fluorescence Exposure:

ICH guidelines specify an exposure of 1.2×106 lux hours for fluorescence (1× ICH). Recommended stress conditions and time points to be tested are 5× and 10× ICH for solid drug substances.

Filters may be useful to determine any wavelength range causing instability. For example, a 400 nm filter in fluorescence experiments to look at the 400–700 nm component and eliminate the UV portion is especially critical in solution experiments. If photodegradation is observed, one can determine whether it is the result of visible or ultraviolet light using appropriate spectral filters.

Table 7. Guidance for Fluorescence Degradation Experimental Set Up:

Samples:	Fluorescence Thermal foil-wrapped control
Time points:	5× and 10 × ICH*

* Note: ICH fluorescent conditions = 1.2×10^6 lux hrs

3.2.Degradation Prediction:

Degradation prediction is extremely helpful for understanding a degradation mechanism. One program that has been particularly useful is CAMEO (computer assisted mechanistic evaluation of organic reactions). CAMEO is a computer program that predicts the products of organic reactions given starting materials, reagents, and conditions. The analyses cover the key degradation conditions, basic/nucleophilic, acidic/electrophilic, radical, oxidative/ reductive and photochemical, as well as mechanistic interpretations of these reactions.

CHAPTER -2

LITERATURE REVIEW

Chapter 2

LITERATURE REVIEW

Shende et al.[28] developed an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method for analysis of irinotecan HCl and validated. Separation was achieved on a C18 column with potassium dihydrogen phosphate buffer (pH adjusted to 3.5 with orthophosphoric acid)–acetonitrile–methanol 55:25:20 (*v*/*v*) as mobile phase at a flow rate of 1.0 mL min^{-1}. UV detection was performed at 254 nm. The method is simple, sensitive, rapid, and selective, and linear over the range 30–70 μg mL^{-1} for assay of irinotecan HCl. The precision of the assay method was below 1.0% RSD. Mean recovery was in the range 98–102%. Recovery of the active pharmaceutical ingredient from dosage forms ranged from 99.0 to 101.0. The method is useful for quality control in bulk manufacture and of the pharmaceutical formulation.

Satyanarayana et al.[29] have reported a reverse phase HPLC method, developed for the estimation of Irinotecan HCl in tablet dosage form. An X-Terra RP C18, 250 X 4.6 mm i.d, 5 m particle size, with mobile phase consisting of Methanol and 0.01 M Ammonium Acetate containing 0.1% formic acid and methanol in the ratio of 50:50 v/v was used. The flow rate was 1 mL/min and the effluents were monitored at 250 nm. The retention time was 4.69 min. The detector response was linear in the concentration of 120-360 mcg/mL. The respective linear regression equation being Y=166582.24x+86439.5. The limit of detection and limit of quantification was 0.06 and 0.18 mcg/mL respectively. The percentage assay of Irinotecan HCl was 99.09 %. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Irinotecan HCl in bulk drug and in its pharmaceutical dosage form.

Venkateswara rao et al.[30] developed a reverse phase high performance liquid chromatography (RP-HPLC) method and validated for quantitative determination of irinotecan in bulk drug samples and formulations. Irinotecan was analyzed by using reverse phase cyano column (4.6 mm x 25 cm, 5 microns), with mobile phase consisting of phosphate buffer: acetonitrile (75:25 v/v), pH adjusted to 2.5 with phosphoric acid. The flow rate was set 0.8 mL/min and the analysis

was performed at wavelength 225 nm using Photo Diode Array (PDA) detector at ambient temperature. The method was validated and stability studies were conducted under different conditions. The retention time for irinotecan was around 5.82 minutes. The calibration curves were linear ($R^2 \geq 0.9998$) over a concentration range from 20.0 to 80.0 µg/mL. Limit of detection (LOD) and Limit of quantitation (LOQ) were 8 ng/mL and 24 ng/mL respectively. The developed method was successfully applied to estimate the amount of irinotecan in injection formulations.

Owens et al.[31] developed a method for the determination of CPT-11 and the three metabolites SN-38, SN-38G, and APC in biological matrices. In most of these published methods, chromatography was carried out with fluorescence detection. Liquid chromatography-mass spectrometry methods have also been described, but despite the latter methods providing good results, the instrumentation involved is expensive and not always readily available for routine drug monitoring.

Takahashi et al.[32] found that both CPT-11 and SN-38 were detectable in saliva and that the patterns of their concentration-time curves in plasma and saliva were very similar. To date, however, no bioanalytical assay has been validated for the determination of irinotecan and its metabolites in saliva. This report describes rapid, specific, reliable, and sensitive analytical methods to simultaneously quantify irinotecan and four metabolites (SN-38, SN-38G, APC, and NPC) in human plasma and saliva. These methods, involving the use of an internal standard, were validated according to validation procedures, parameters, and acceptance criteria based on US Pharmacopoeia XXIII guidelines and Food and Drug Administration guidance.

Ali mohammadi et al.[33] developed a new simple high performance liquid chromatography (HPLC) method and validated for the simultaneous determination of irinotecan (CPT-11) and two related compounds *viz*., 7-ethyl-10-hydroxycamptothecin (SN-38) and camptothecin (CPT) in pharmaceutical dosage forms. Chromatography was accomplished using a reversed-phase C18 column and ultraviolet (UV) detection and an isocratic mobile phase consisting of 3 % v/v triethylammonium acetate buffer (pH 3) and acetonitrile (70:30 v/v). The linear range of

quantitation for all the compounds was 0.1-10 µg/mL. The limit of quantitation for all the compounds ranged between 0.01-0.05 µg/mL. The method has the requisite accuracy, selectivity, sensitivity and precision to assay of CPT-11 and related compounds in pharmaceutical dosage forms and bulk API.

Mohammed Ishaq et al.[34] have reported a rapid and sensitive RP-HPLC method with UV detection at 222 nm for routine analysis of Irinotecan in bulk and pharmaceutical formulations. Chromatography was performed with mobile phase containing a mixture of acetonitrile and phosphate buffer in the ratio of 60:40, v/v with flow rate 1.0 mL/min. The calibration graph of Irinotecan was found to be linear over the range of 40 to 120 µg/mL with correlation coefficient of 0.9999. Sensitivity, accuracy, range, precision, robustness, ruggedness, stability, specificity, limit of detection, limit of quantification and system suitability parameters were validated for the developed method. The developed method was successfully applied to estimate the amount of irinotecan in injection formulations.

Gogineni Ratna Prasad et al.[35] developed a simple, specific, accurate and precise reverse phase high performance liquid chromatographic method and validated for the estimation of Irinotecan in tablet dosage form. An Inertsil ODS C-18, 5 µm column having 250 x 4.6 mm internal diameter in isocratic mode with mobile phase containing Methanol: Triethylamine: 1% Orthophosphoric acid in the ratio of 60:5:35 (v/v/v) was used. The flow rate was 1.0mL/min and effluents were monitored at 254 nm. The retention time for Irinotecan was 5.109 min. The method was validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. Limit of detection and limit of quantification were found to be 0.09 ppm and 0.297 ppm respectively. The proposed method was successfully applied for the quantitative determination of Irinotecan in tablet formulation.

Saini P.K et al.[36] proposed a selective and highly sensitive reverse phase ultra performance liquid chromatographic (UPLC) method and validated for the quantitation of the novel anticancer agent irinotecan hydrochloride in Bulk and its injection dosage form. The method utilizes sample preparation step followed by separation on a waters BEH $C_{18,}$ 50 × 2.1 mm, 1.7 µm particle size

column in gradient mode using 0.1 % v/v orthophosphoric acid in water and acetonitrile as the mobile phase. The analytical column was thermo stated at 50°C and flow rate was set at 0.5 mL per min. The eluted peaks were detected by photodiode array (PDA) detector at a wavelength of 254 nm. The retention time of irinotecan was found 2.127 min. Linearity responses in analyte standard peak areas were observed over the concentration ranges of 100 - 500 µg/mL. The limit of detection and limit of quantification were found 0.0560 and 0.1698 µg/mL, respectively. Percentage recoveries were obtained in the range of 97.5 % and 98.88 %. The proposed method is precise, accurate, selective and reproducible.

Sushama Talegaonkara et al.[37] developed a simple, sensitive, precise and accurate stability-indicating high performance thin-layer chromatographic method for analysis of irinotecan both as bulk drug and marketed injectables and validated as per ICH guidelines. Chromatographic separation was achieved on LiChrospher aluminum plates precoated with silica gel 60F254 as stationary phase. The solvent system consisted of acetone –ethyl acetate –acetic acid 8.5:1.5:0.1 (v/v/v) and this system were found to give compact spots for irinotecan at Rf value of 0.31 ± 0.02. Densitometric analysis was performed in the absorbance at 366 nm. The linear regression analysis for the calibration plots showed good linear relationship with R^2 = 0.9973 ± 0.0013 in the concentration range of 50–500 ng/spot. The % recovery (94.63–101.40%) and precision (≤ 4.30) were found to be satisfactory. Irinotecan was subjected to acid and alkali hydrolysis, oxidation, thermal and ultraviolet radiation treatments. All the peaks of degradation products were well resolved from the standard drug with significantly different retention factor (Rf) values. Developed method effectively separated out the drug from its degradation products and hence can be used as stability-indicating method as well as in routine analysis of irinotecan.

Wei Zhang et al.[38] developed a sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for the determination of Irinotecan (CPT-11) and its metabolites in human plasma. Samples were prepared after protein precipitation and analyzed on a C_{18} column interfaced with a Q-Trap tandem mass spectrometer. Positive electrospray ionization was employed as the ionization source. The mobile phase consisted of acetonitrile–water (0.05% formic acid), using gradient procedure. The analytes and internal standard camptothecin were both detected by use of multiple reaction monitoring mode. The method was

linear in the concentration range of 10.0–2000.0 ng/mL for CPT-11 and 0.5–200.0 ng/mL for 7-ethyl-10-hydroxycamptothecin (SN-38), respectively. The lower limit of quantification (LLOQ) was 10 ng/mL for CPT-11 and 0.5 ng/mL for SN-38. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 10.6%. The accuracy determined at three concentrations was within ± 11.4% in terms of relative error. Due to the unavailability of standard for 7-ethyl-10-*O*-glucuronyl-camptothecin (SN-38G) and the importance of knowing the concentration of this metabolite, we developed a method for analysis SN-38G by taking advantage of the quantitative conversion of SN-38G to SN-38 using glucuronidase. This enzymatic method of identification and quantitation of gluconated compound can be widely used when the standard for phase II glucuronide metabolites are not available.

Iman Barilero et al.[39] have reported A sensitive high-performance liquid chromatographic assay suitable for the simultaneous determination of irinotecan HCl (I) and its active metabolite SN-38 (II) in human plasma, and their preliminary clinical pharmacokinetics, are described. Plasma samples were processed using a solid-phase (C_{18}) extraction step allowing mean recoveries of I, II and the internal standard camptothecin (III) of 84, 99 and 72%, respectively. The extracts were chromatographed on a C_{18} reversed-phase column with a mobile phase composed of acetonitrile, phosphate buffer and heptanesulphonic acid, with fluorescence detection. The calibration graphs were linear over a wide range of concentrations (1 ng/mL-10 μg/mL), and the lower limit of determination was 1 ng/mL for both I and II. The method showed good precision: the within-day relative standard deviation (R.S.D.) (5- l000 ng/mL) was 13.0% (range 4.9-19.4%) for I and 12.8% (6.7- 19.1%) for II; the between-day R.S.D. (5-10 000 ng/mL was 7.9% (5.417.5%) for I and 9.7% (3.515.1%) for II. Using this assay, plasma pharmacokinetics of both I and II were simultaneously determined in three patients receiving 100 mg/m^{2}I as a 30-min intravenous infusion. The mean peak plasma concentration of I at the end of the intravenous infusion was 2400 ±285 ng/mL (mean ± standard error of the mean). Plasma decay was triphasic with half-lives α, β and ∞ of 5.4 f 1.8 min, 2.5 f 0.5 h and 20.2 f 4.6 h, respectively. The volume of distribution at steady state was 105 ± 15 l/m^2, and the total body clearance was 12.5 ± 1.9 l/h m^2. The maximum concentrations of the active metabolite II reached 36 f 11 ng/mL.

CHAPTER -3

Drug profile

Chapter 3

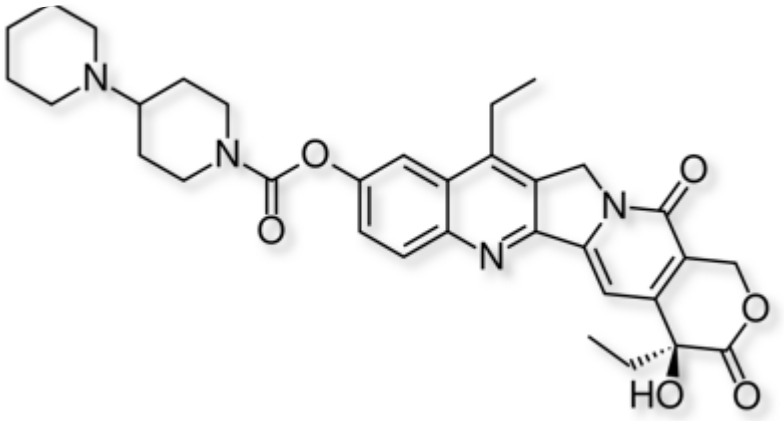
DRUG PROFILE [25-27]

IRINOTECAN HYDROCHLORIDE

Irinotecan HCl also called as Irinotecanum available in market in various following Brand names

- Camptosar
- CP0
- Irinotecan
- CPT-11
- Irinocam
- Torsin
- campto

Structure:



.HCl .3H$_2$o

Molecular formula:

$C_{33}H_{38}N_4O_6 . HCl\ 3H_2O$

Molecular weight:

Trihydrate: 677.18

Anhydrous: 623.14

Chemical name:

(S)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3, 14-dioxo-1H- Pyranol-[3`, 4`:6,7]-indolizino [1,2-b]quinolin-9-yl-[a,4`-bipiperidine]-1`-carboxylate, monohydrochloride, trihydrate.

Content:

Irinotecan Hydrochloride contains NLT 98.0% and NMT 102.0% of $C_{33}H_{38}N_4O_6 \cdot HCl$, calculated on the anhydrous basis.

Characteristics:

Appearance: Pale yellow crystalline powder

Solubility: Slightly soluble in water, methanol and ethanol, **freely soluble** in glacial acetic acid.

Identification:

IR

Melting/Freezing Point: 222-223^{o}C

Boiling Point: 100°C

P^{H}: 3.5

Mechanism of action:

Irinotecan inhibits the action of topoisomerase I. Irinotecan prevents religation of the DNA strand by binding to topoisomerase I-DNA complex. The formation of this ternary complex interferes with the moving replication fork, which induces replication arrest and lethal

double-stranded breaks in DNA. As a result, DNA damage is not efficiently repaired and apoptosis (programmed cell death) occurs.

Toxicity:

Gastrointestinal complications such as nausea, vomiting, abdominal cramping, diarrhea, and infection.

Storage:

Stored in Glass containers, Polyethylene or polypropylene containers at 15-30^{o}C, and protected from light.

Category:

- Antineoplastic Agents
- Radiation-Sensitizing Agents
- Parasympathomimetics
- Enzyme Inhibitors
- Prodrugs
- Phytogenic agent
- Extreme suppression of the immune system.

Metabolic pathway of Irinotecan HCl:

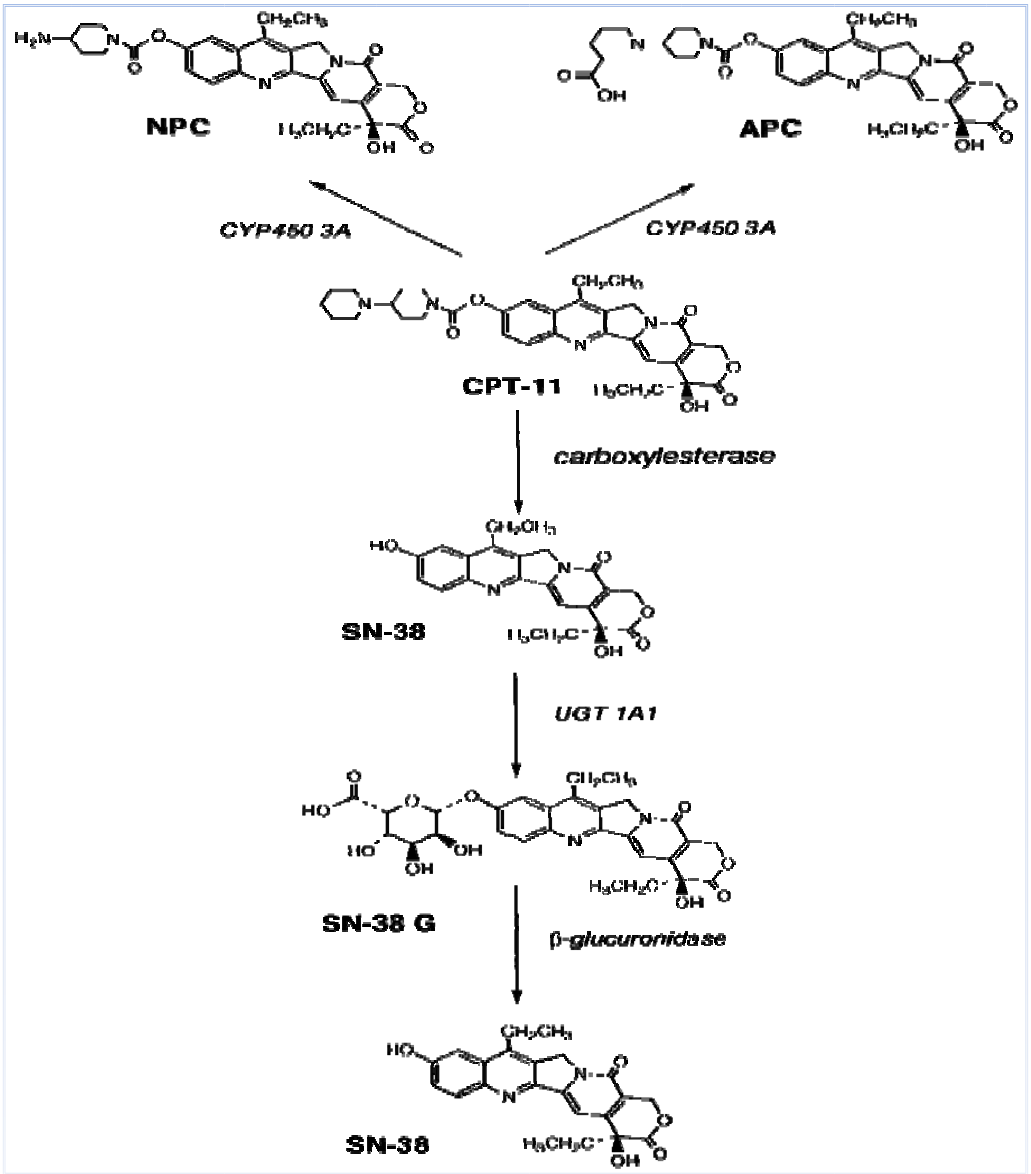


Fig. 2

Metabolic pathways of CPT-11 showing CE-mediated formation of the active metabolite SN-38 and its subsequent conversion to a glucuronide derivative (SN-38G) by UGT1A1 and 1A7 isoforms (UGT1A7), with deglucuronidation by intestinal β-glucuronidase (*β-Glu*). CPT-11 can also undergo CYP3A4-mediated oxidative metabolism to form APC and NPC, of which the latter can be hydrolyzed by CE to release SN-38.

CHAPTER -4

AIM & OBJECTIVE

Chapter 4

AIM AND OBJECTIVE

SN-38 (7-ethyl-10-Hydroxycamptothecin) is one of the metabolite/related substance present in irinotecan HCl [40-41]. The assessment of the limit of SN38 concentrations in humans is very important, since SN38 is 100 to 1,000 fold more cytotoxic than the parent compound. SN-38 is reported to be associated with the severe diarrhea with one of the major side effects causing direct enteric injury (Araki et al., 1993). There is no report for the quantification of SN-38 in irinotecan HCl and on forced degradation studies of irinotecan HCl by HPLC method.

Hence it was proposed to develop and validate a RP-HPLC method for detection and quantification of SN-38 in irinotecan HCl and to perform the forced degradation studies of irinotecan HCl in order to identify and quantify the degradents by HPLC.

CHAPTER -5

PLAN OF WORK

Chapter 5

PLAN OF WORK

- **PHASE -I**

1. Optimization of chromatographic conditions,

- ❖ Selection of wavelength
- ❖ Selection of initial separation conditions
- ❖ Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- ❖ Nature of the stationary phase
- ❖ Selection of separation method and agent

- **PHASE-II**

2. Validation of the method

The developed method to be validated using the various validation parameters such as,

- ❖ Accuracy
- ❖ Precision
- ❖ Linearity and of detection (LOD) / Limit of quantitation (LOQ)
- ❖ Selectivity / Specify
- ❖ Robustness / ruggedness
- ❖ System suitability.

- **PHASE-III**

3. Quantification of Related substance:

Estimation of Related substance (SN-38) in marketed Irinotecan HCl formulation and verification of its limits

- **PHASE-IV**

4. Performance of forced degradation studies such as

- Acid degradation
- Alkaline degradation
- Oxidative degradation
- Photolytic degradation
- Thermal degradation

CHAPTER -6

EXPERIMENTAL

Chapter 6

EXPERIMENTAL

6.1 Analytical Method Development and determination of Irinotecan HCl and its related substance 7-ethyl 10-hydroxy camptothecin (SN-38) in marketed formulation

6.1.1 MATERIALS AND INSTRUMENTS USED

a) Drug sample & Study products

Irinotecan HCl was obtained from Dr. Reddy's Laboratories, Hyderabad and 7-ethyl 10-hydroxy camptothecin (SN-38) was obtained from Henan Dongtai Pharm Co., Ltd, China.

Test product:

Irinocam-40 mg (Dr. Reddy's Laboratories) injection was purchased from the local market.

b) Chemicals and solvents used for estimation:

- HPLC grade Water
- HPLC grade Acetonitrile
- HPLC grade Methanol
- Analytical grade Dihydrogen sulphoxide
- Analytical grade Trichloro acetic acid
- Analytical grade Distilled water

c) Instruments used:

- Elico pH meter LI 127.
- Shimadzu LC-20 AT HPLC.
- Shimadzu 1700 UV Spectrophotometer.
- Sonica Ultrasonic cleaner.
- Solvent filtration unit – Millipore.
- Shimadzu electronic balance AX 200.

D) preparation of standard stock solutions:

I. Irinotecan HCl (10 µg):

Ten milligrams (mg) of standard Irinotecan HCl was dissolved in the 10 mL of methanol, then serial dilutions were made with water to get a final concentration of 10 µg/mL.

II. SN-38 (7-ethyl 10-hydroxy camptothecin) 10 µg:

Ten milligrams (mg) of standard SN-38 was dissolved in the 10 mL of Dihydrogen sulphoxide, then serial dilutions were made with methanol to get a final concentration of 10 µg/mL.

6.1.2 OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR METHOD DEVELOPMENT OF IRINOTECAN HCl AND SN-38 (7-ETHYL 10-HYDROXY CAMPTOTHECIN)

Method development:

The aim of the present study was to develop and validate the RP-HPLC method for the estimation of Irinotecan HCl and SN-38 in the injection dosage form.

Selection of wavelength

An UV-Visible spectrum of 10 µg/mL Irinotecan HCl in methanol and SN-38 in Dihydrogen sulphoxide and methanol was recorded by scanning in the range of 200 nm to 800 nm. From the UV-Visible spectrum wavelength of 372 nm was selected. At this wavelength Irinotecan HCl showed good absorbance.

Selection of chromatographic method

Proper selection of the method depends upon the nature of the sample (ionic/ionisable/neutral molecule), its molecular weight and solubility. The drug selected in the present study is polar in nature and hence a reverse phase HPLC was selected for the initial separations because of its simplicity and suitability.

Initial separation conditions

Standard solution: 10 µg/mL of Irinotecan HCl in HPLC grade Methanol.

Equipment

System : Shimadzu gradient HPLC

Pump : LC – 20AT prominence solvent delivery system

Detector : SPD-M20A Prominence Diode array detector

Injector : Rheodyne 7725*i* with 20 µL loop

Chromatographic condition 1

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 10 mM ammonium acetate in 100mL HPLC grade water P^H adjusted to 4 with orthophosphoric acid

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 60:40

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

The retention time of Irinotecan HCl andSN-38 were found to be 6.67 and 8.75 min but peak tailing was observed for Irinotecan HCl as shown in **Fig. 3 &4**. So we adjusted the P^H of the buffer solution.

Chromatographic condition 2

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 10 mM ammonium acetate in 100 mL HPLC grade water P^H adjusted to 5.0 with orthophosphoric acid

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 60:40

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

Retention time for irinotecan HCl was found to be 8.82 and for SN-38 we found 13.3 min but splitting and peak tailing was observed for SN-38 as shown in **Fig. 5 & 6**. So we altered the ratio of the mobile phase system and run for SN-38.

Chromatographic condition 3

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 10 mM ammonium acetate in 100 mL HPLC grade water P^H adjusted to 4.5 with orthophosphoric acid

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 55:45

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

Retention time for SN-38 was found to be 6.8 min and peak splitting was observed with tailing as shown in **Fig. 7**. Then we altered the buffer system.

Chromatographic condition 4

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 0.5% Trichloro acetic acid in 100 mL of HPLC grade water

Solvent B: methanol

Solvent ratio : 55:45

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

Retention time for SN-38 was found to be 13.8 min but broad peak was obtained as shown in **Fig. 8**. Then we altered the organic phase.

Chromatographic condition 5

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 0.5% Trichloro acetic acid in 100 mL of HPLC grade water

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 55:45

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

Both Irinotecan HCl and SN-38 were merged and gave retention time at 5.8 min in individual chromatograms as shown in **Fig. 9 & 10**.

Chromatographic condition 6

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 0.5% Trichloro acetic acid in 100 mL of HPLC grade water

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 60:40

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

Retention time for Irinotecan HCl and SN-38 were found to be 8.53 and 7.25 min with good resolved peaks as shown in **Fig. 11 & 12**.

Chromatographic condition 6 for mixture:

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 0.5% Trichloro acetic acid in 100 mL of HPLC grade water

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 60:40

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL (mixture of both)

Needle wash : water HPLC grade

Column temperature : Ambient

Retention time for Irinotecan HCl and SN-38 were found to be 8.53 and 7.25 min with good resolved peaks in chromatogram without merging as shown in **Fig. 13**.

Effect of ratio of mobile phase

Aqueous and organic mixture with 60:40 and 55:45 ratios were used as the mobile phase. At 60:40 ratio, the symmetric peaks were eluted at 8.63 and 7.35 min. At 55:45 ratio the peaks were asymmetric. Thus for the present study 60:40 ratio mobile phase system was selected.

Finally, the following Fixed Chromatographic Conditions were selected for the estimation of Irinotecan HCl and its related substance (SN-38) in marketed formulation.

Stationary phase : Phenomenex Luna C_{18} Column

Mobile phase : *Solvent A*: 0.5% Trichloro acetic acid in 100 mL of HPLC grade water

Solvent B: Acetonitrile and methanol (50:50 v/v)

Solvent ratio : 60:40

Detection : 372 nm

Flow rate : 1.0 mL/min

Sample size : 20 µL

Needle wash : water HPLC grade

Column temperature : Ambient

6.1.3 ESTIMATION OF IRINOTECAN HCL AND ITS RELATED SUBSTANCE (SN-38) IN MARKETED FORMULATION:

Preparation of standard solutions:

I. **Irinotecan HCl (10 µg):**
Ten milligrams (mg) of standard Irinotecan HCl was dissolved in the 10 mL of methanol, then serial dilutions were made with water to get a final concentration of 10 µg.

II. **SN-38 (7-ethyl 10-hydroxy camptothecin) 10 µg:**
Ten milligrams (mg) of standard SN-38 was dissolved in the 10 mL of Dihydrogen sulphoxide, then serial dilutions were made with methanol to get a final concentration of 10 µg.

Preparation of test (marketed formulation) solution:

One milliliter (mL) of formulation (20 mg) was diluted to 10 mL of methanol and then serial dilutions were made with methanol to get final concentrations of 60 and 120 µg.

Recording of chromatogram:

With the optimized chromatographic conditions mentioned above, a steady baseline for about 20 min was recorded. After the stabilization of the baseline, the standard solution were injected and chromatograms were recorded until the reproducibility of the peak areas were found satisfactory and finally 10 µg/mL of the standard solution of the individual samples of Irinotecan HCl and SN-38 and Standard solutions containing 1-5 µg /mL of Irinotecan HCl and SN-38 were injected and chromatograms were recorded. Retention time of Irinotecan HCl and SN-38 were found to be at 8.6 and 7.3 min respectively and the chromatograms are shown in **Fig. 14, 15, 16, 17, 18, 19.**

The procedure was repeated for sample (formulation) solution and the chromatogram is recorded as shown in **Fig. 20, 21, 22, 23.** Peak areas of the sample chromatograms were recorded and the amount of Irinotecan HCl and SN-38 were calculated from regression equation.

6.1.4 VALIDATION OF THE HPLC METHOD

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics**.**

Significance of Method Validation:

The quality of analytical data is a key factor in the success of a drug development program. The process of method development and validation has a direct impact on the quality of these data.

Analytical validation is a very important feature of any package of information submitted to international regulatory agencies in support of new product marketing or clinical trials applications. A thorough method development can almost rule out all potential problems, at the same time, a thorough validation programmed can address the most common ones and provide assurance to the intended purpose (can be used with 100% confidence). In other words, a thorough validation can fulfill all the technical and regulatory objectives. A direct consequence and most significant out come from any method validation exercise is the development of meaningful specifications can be predicted upon the use of validated analytical procedures that can assess changes in a drug substance or drug product during its life time.

Analytical characteristics listed below may not be applicable to every test procedure or every particular material. It will mostly depend on the purpose for which the procedure is required, however, these following aspects of validation should be given due importance.

a) Accuracy:

Accuracy of the method was determined by recovery experiments. The reference standards of the respective drug were added to the sample solution at the level of 50%, 100% and 150%. These were further diluted by procedure as followed in the estimation of formulation. The concentrations of the drugs present in the resulting sample solution were determined by using validated assay method.

b) Precision:

Repeatability studies were done by consequently injecting the standard solution of same concentration i.e., 120 µg/mL of Irinotecan HCl. These solutions were prepared in duplicate and injected as per assay procedure.

c) Linearity and Range:

The calibration curve was plotted using peak area ratio Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated using linear regression equation.

d) Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD and LOQ were separately determined, based on the calibration curve of standard solution. The residual standard deviation of the regression line or the standard deviation of y – intercepts of regression lines may be used to calculate LOD and LOQ. LOD = 3.3 × D/S and LOQ = 10 × D/S, where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve.

e) Specificity

The specificity of the RP-HPLC method was determined by complete separation of Irinotecan HCl with parameters like retention time (R_t), resolution (Rs) and tailing factor (T). Tailing factor for peaks of Irinotecan HCL was less than 2% and resolution was satisfactory. The average retention time ± standard deviation for Irinotecan HCL was found to be 8.7 ± 0.468 respectively for six replicates. The peaks obtained for Irinotecan HCL was sharp and have clear baseline separation.

f) System Suitability Studies:

The system suitability studies were carried out as specified in USP. These parameters include column efficiency, resolution and capacity factor.

h) Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

1) ± 2% in ratio of acetonitrile in mobile phase,

2) ± 0.1 mL of flow rate,

3) ± 0.2 units in the pH of buffer.

The separation factor, retention times and peak symmetry were then calculated.

The deviation among the results obtained is well within the limits. Hence the method is rubusted.

6.1.5 FORCED DEGRADATION OR STABILITY INDICATING STUDIES:

The specificity of the method was demonstrated through forced degradation studies conducted on the sample using Acid, alkaline, oxidative, reductive and Photolytic degradations. The sample was exposed to these conditions and the main peak was studied for the peak purity (Irinotecan peak), thus indicating that the method effectively separated the degradation products from the Irinotecan active ingredient.

Acid Degradation:

About 10 mL of the 20 µg/mL of Irinotecan HCl solution (pure) and test solution was transferred to a 50 mL volumetric flask individually. About 10 mL of 1N hydrochloric acid was added. The volumetric flask was placed on a water bath maintained at 60°C for 8 h and cooled then this solution was neutralized with 1 mL of sodium hydroxide solution.

Alkaline Degradation:

About 10 mL of the 20 µg/mL of Irinotecan HCl solution (pure) and test solution was transferred to a 50 mL volumetric flask individually. Ten milliliter (mL) of 1N sodium hydroxide solution was added. The volumetric flask was then placed on a water bath maintained at 60°C for 8 h. Then it was cooled and neutralized with 1 mL of hydrochloric acid.

Oxidative Degradation:

About 10 mL of the 20 µg/mL of Irinotecan HCl solution (pure) and test solution was transferred to a 50 mL volumetric flask individually .10 mL of 3% hydrogen peroxide was added. The volumetric flask was placed on a water bath maintained at 60°C for 8 h then cooled to room temperature.

Photolytic Degradation:

About 50 mL of the 20 µg/mL of Irinotecan HCl solution (pure) and test solution was transferred to a 50 mL volumetric flask individually. Then the solutions were exposed to sun light for 24 h

Thermal (or) Dry heat degradation:

About 100 mg of the irinotecan pure drug sample was taken in Petri dish and kept in a hot air oven at 60°C for 8 h to study the heat degradation. A solution of 20 µg/mL of the dry heat degraded sample was prepared.

In all the forced degradation studies, a volume of 5 mL of each of the solutions from the degradation experiments, were transferred to 10 mL individual volumetric flasks and equal volumes of all the above solutions (20 µL) were injected into the column and the chromatograms were recorded and the chromatograms was shown in **Fig. 24, 25, 26, 27, 28, 29, 30, 31, 32.**

CHROMATOGRAMS AND PEAKS:

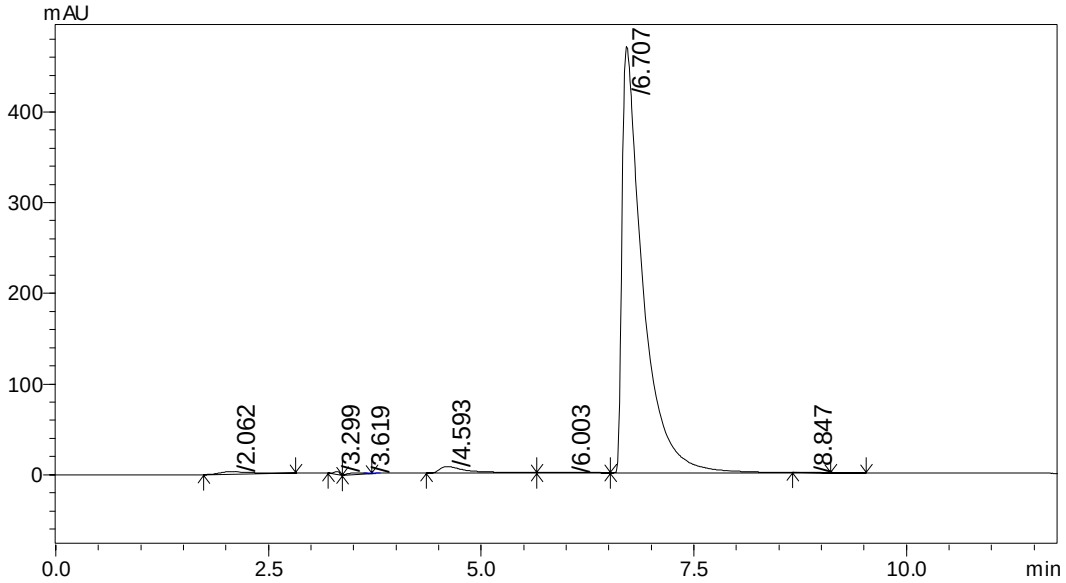


Fig. 3 – Optimization condition 1 for Irinotecan HCl (10 µg/mL)

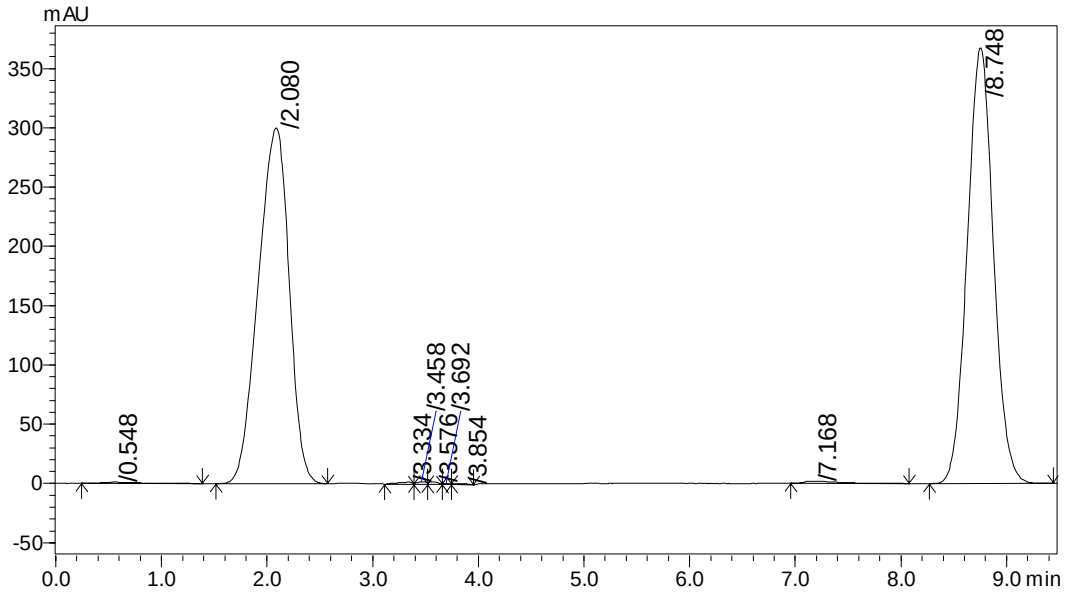


Fig.4- Optimization condition 1 for SN-38 (10 µg/mL)

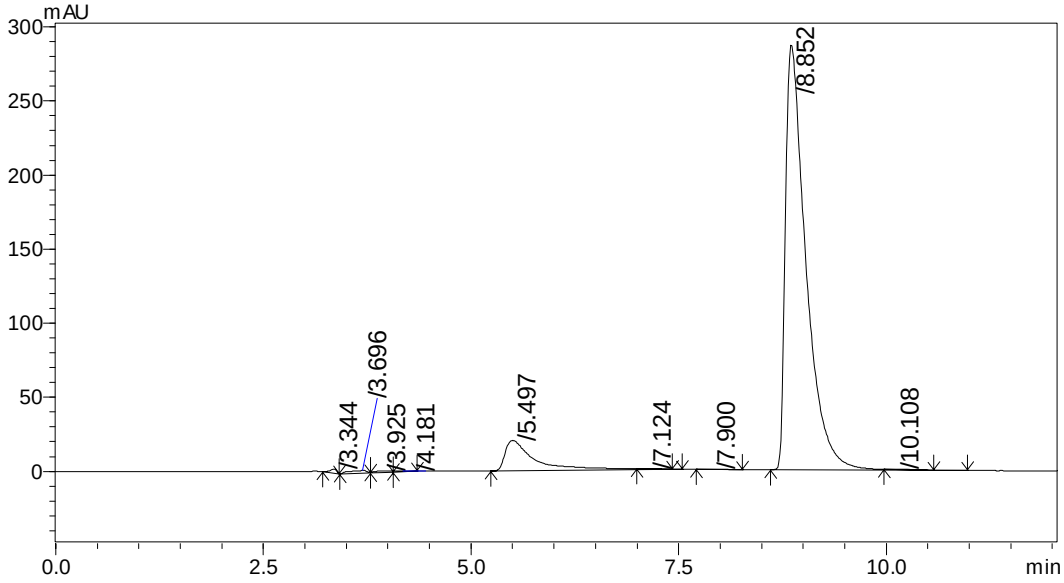


Fig. 5 – Optimization condition 2 for Irinotecan HCl (10 µg/mL)

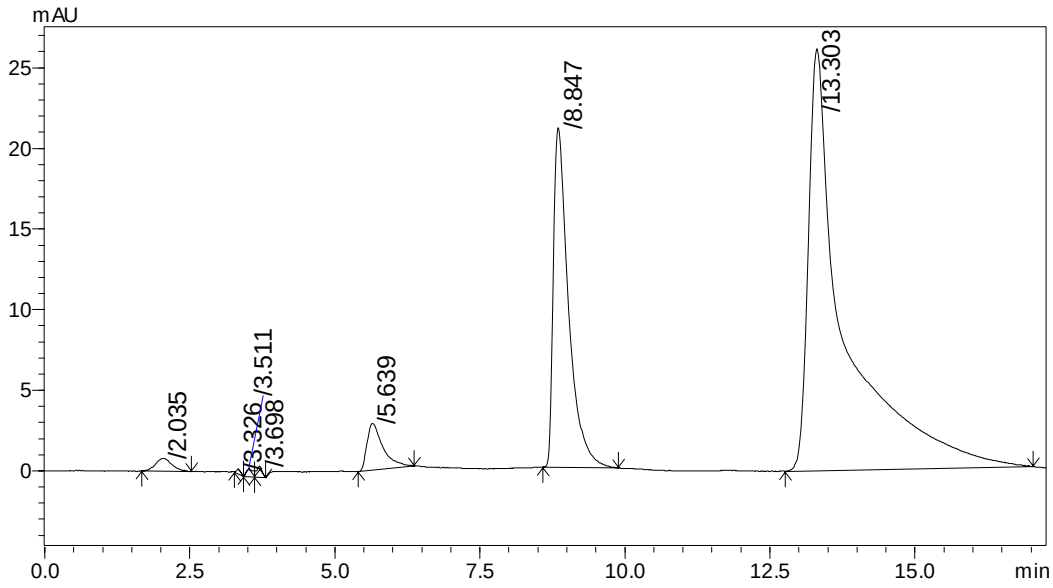


Fig. 6 – Optimization condition 2 for SN-38 (10 µg/mL)

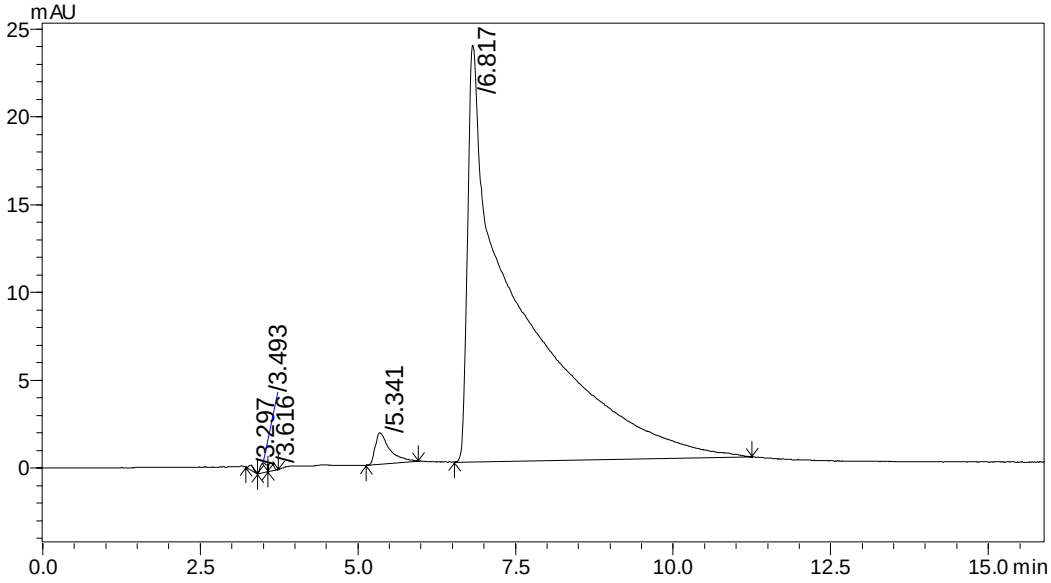


Fig. 7 – Optimization condition 3 for SN-38 (10 µg/mL)

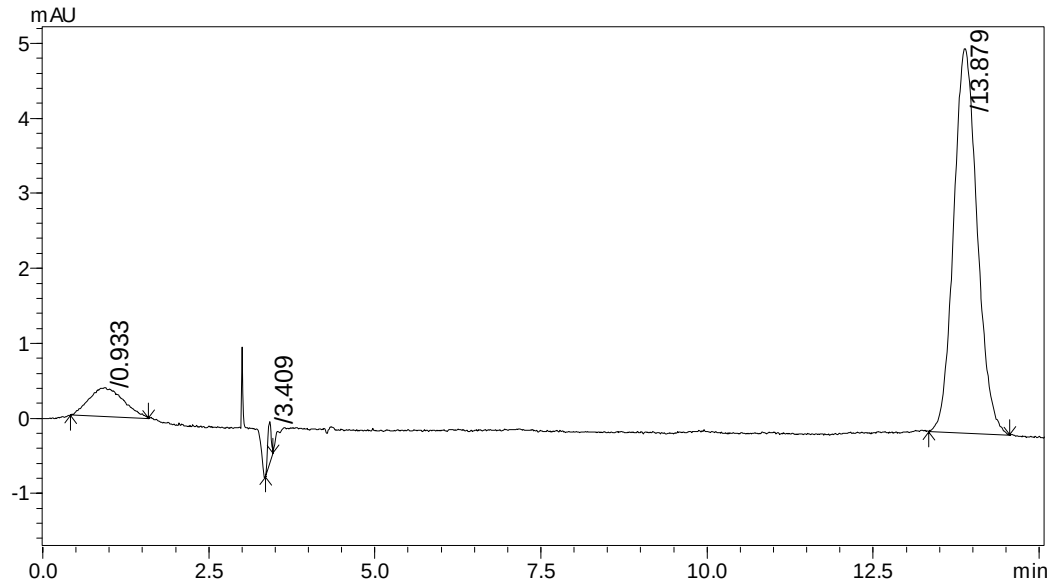


Fig. 8 - Optimization condition 4 for SN-38 (10 µg/mL)

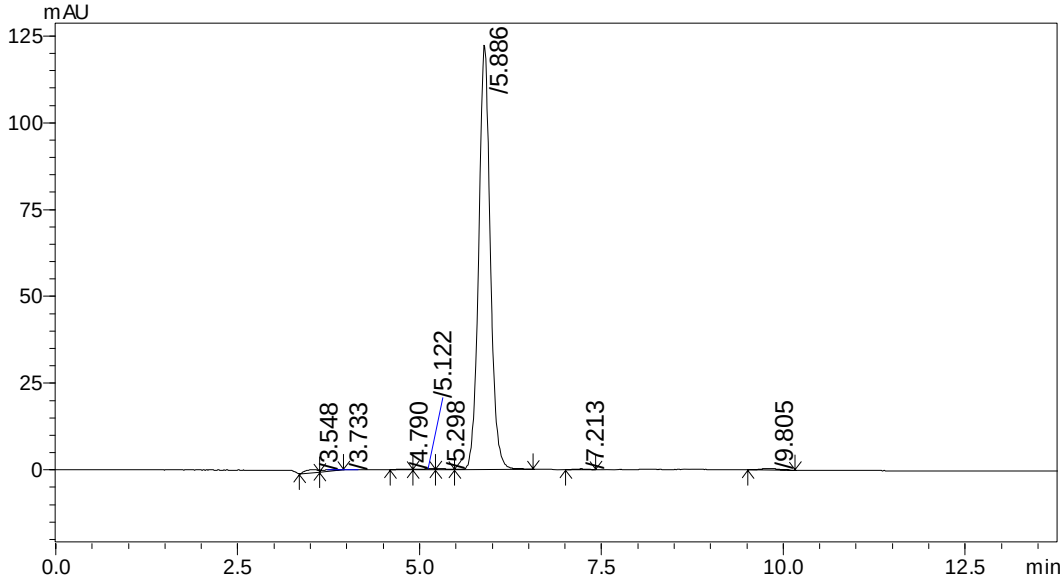


Fig. 9 – Optimization condition 5 for Irinotecan HCl (10 µg/mL)

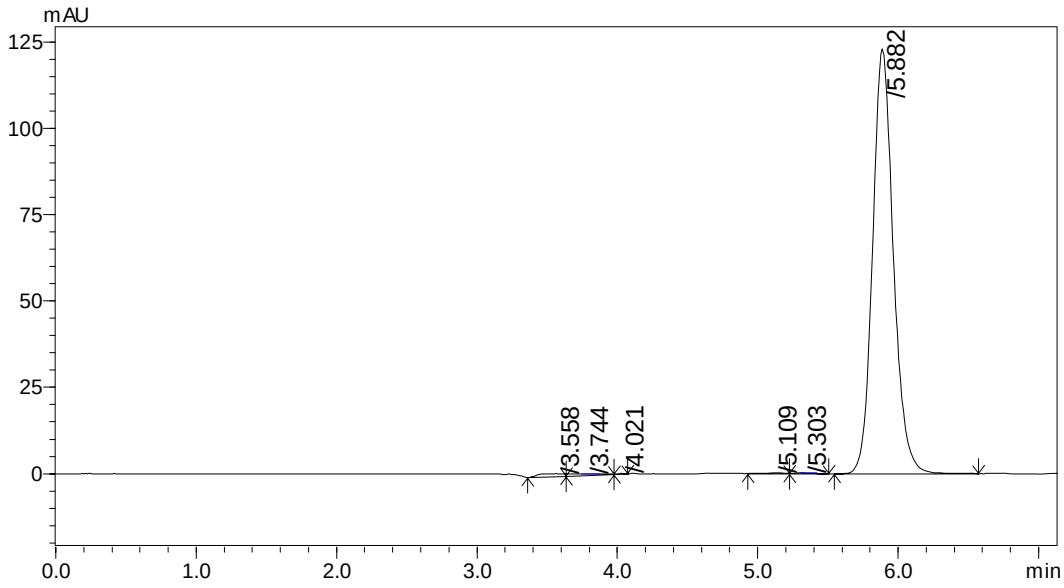


Fig. 10 – Optimization condition 5 for SN-38 (10 µg/mL)

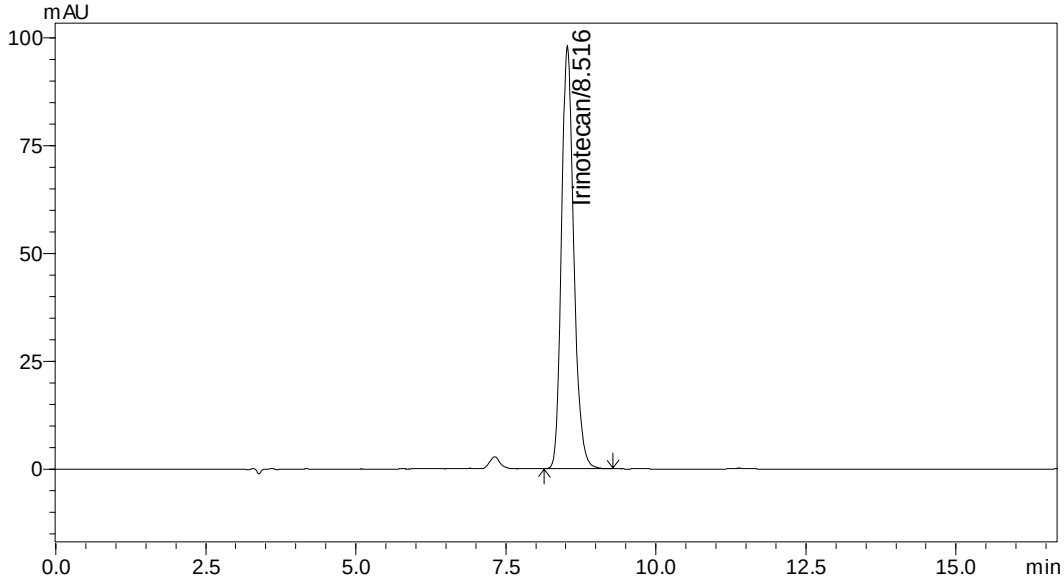


Fig. 11 – Optimization condition 6 for Irinotecan HCl (10 µg/mL)

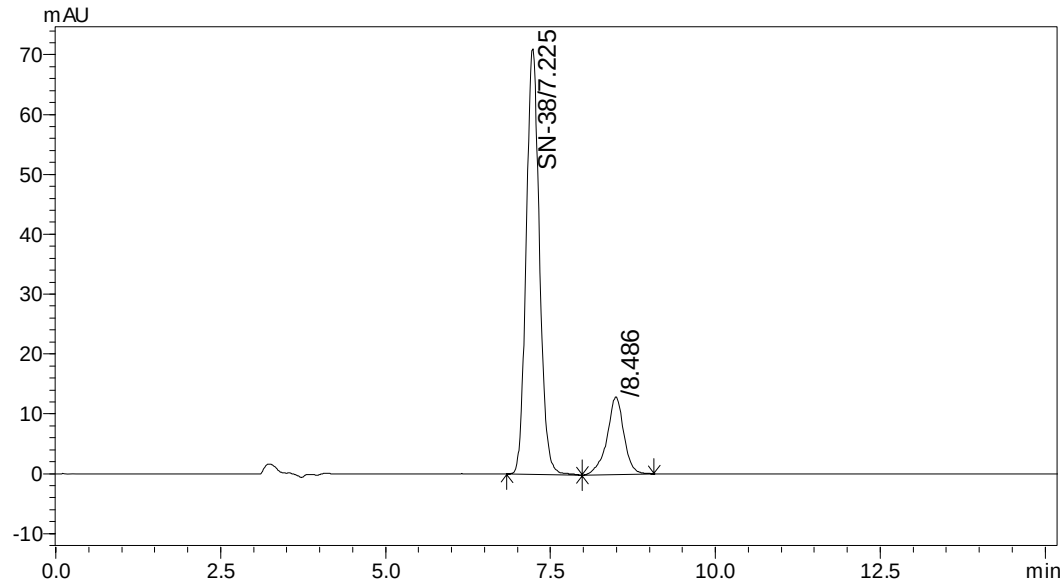


Fig. 12 – Optimization condition 6 for SN-38 (10 µg/mL)

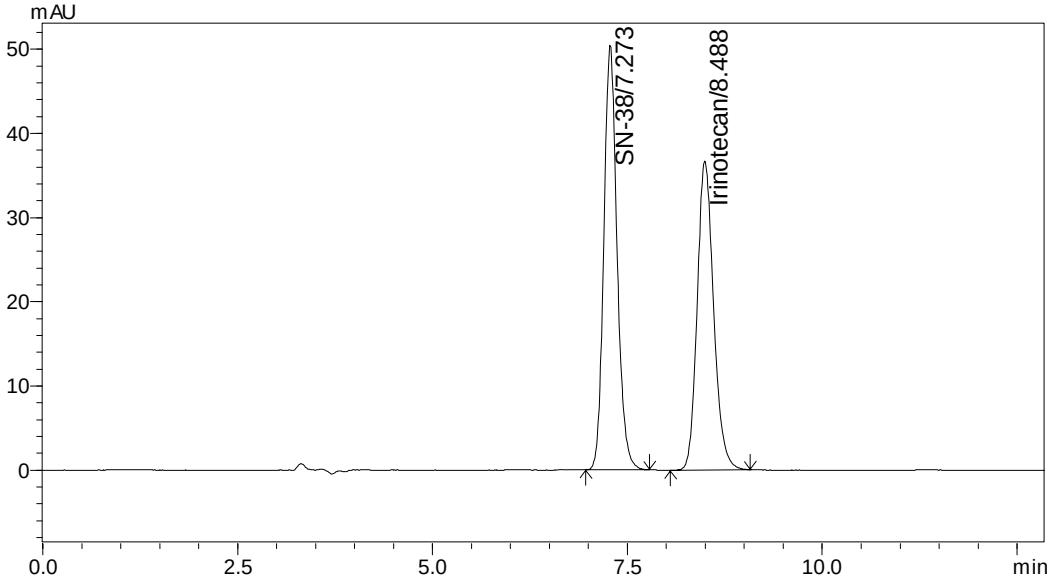


Fig. 13 – Optimization condition 6, mixture of both standards (10 µg/mL)

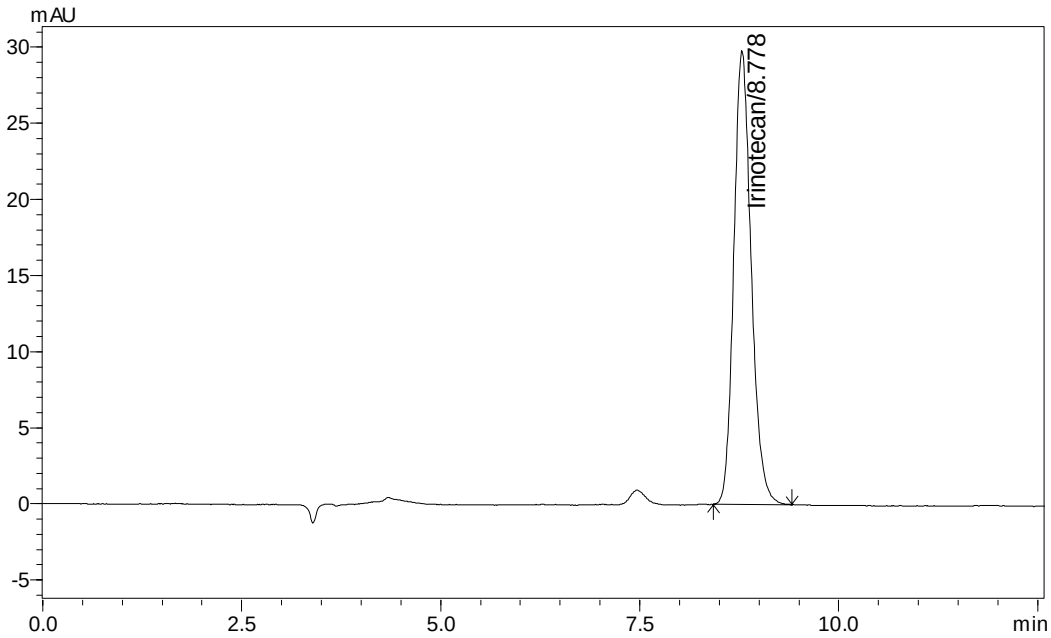


Fig. 14 – HPLC Chromatogram of Standard Irinotecan HCl (10 µg/mL)

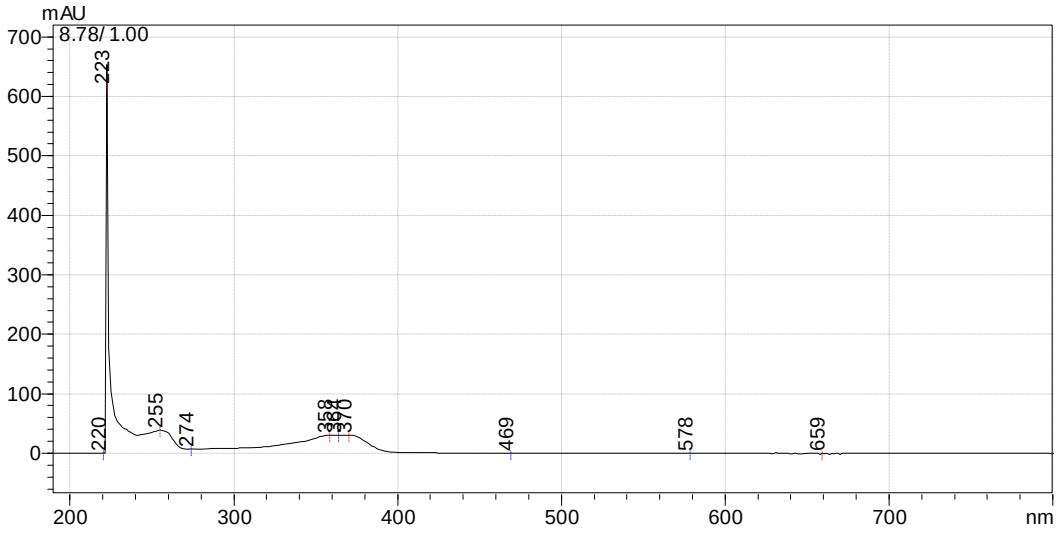


Fig. 15 – UV-spectrum for standard Irinotecan HCl (10 µg/mL)

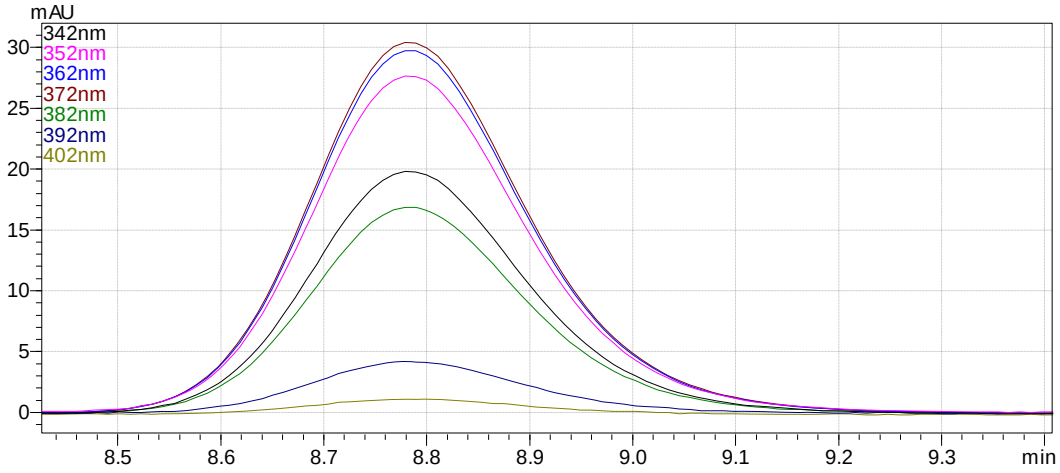


Fig. 16 – Peak profile for standard Irinotecan HCl (10 µg/mL)

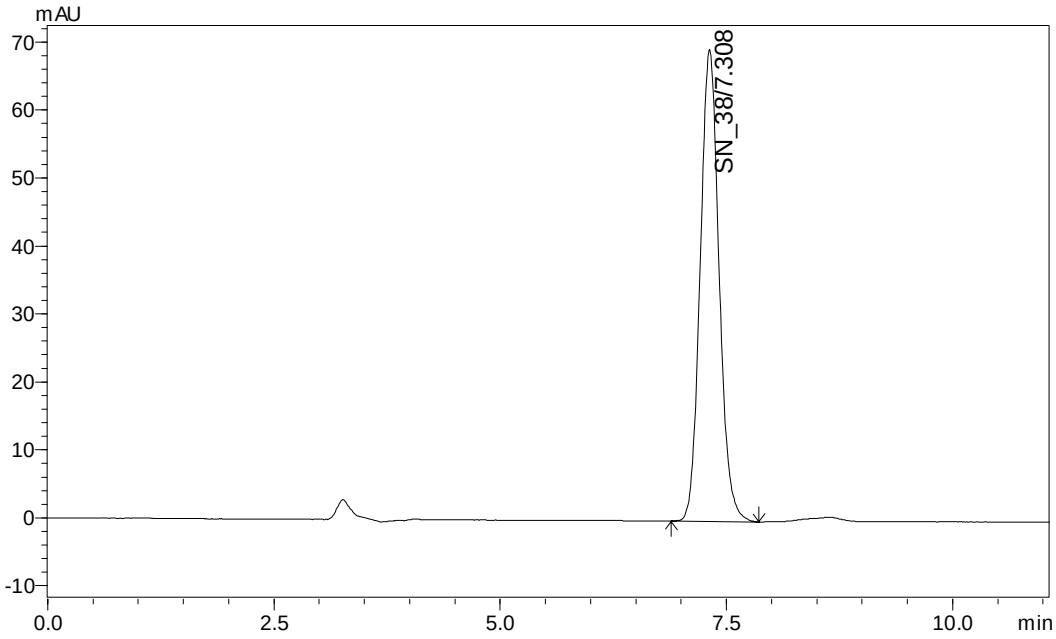


Fig. 17 – HPLC chromatogram for standard SN-38 (10 µg/mL)

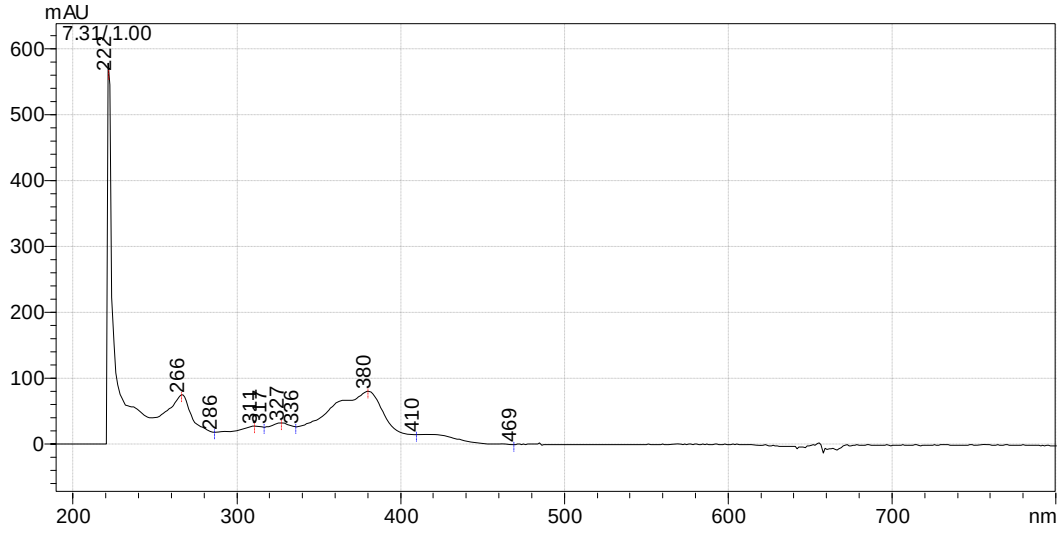


Fig. 18 – UV-spectrum for SN-38 (10 µg/mL)

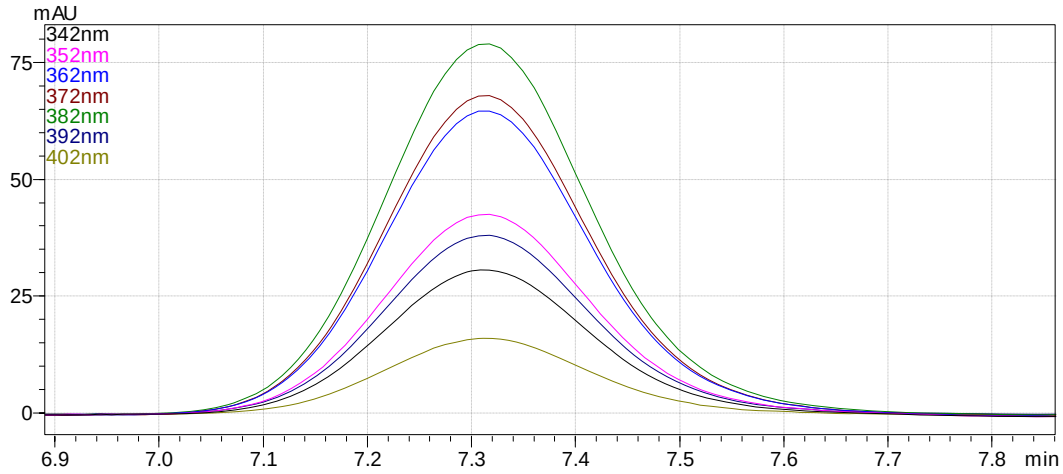


Fig. 19 – Peak profile for Standard SN-38 (10 µg/mL)

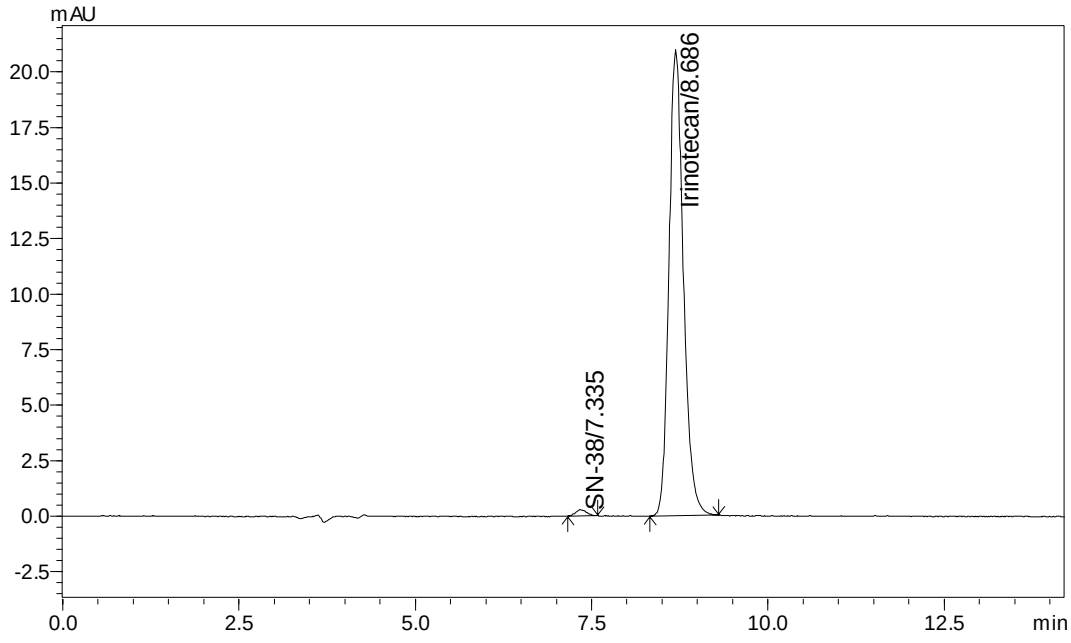


Fig. 20 – HPLC chromatogram for marketed formulation (60 µg/mL)

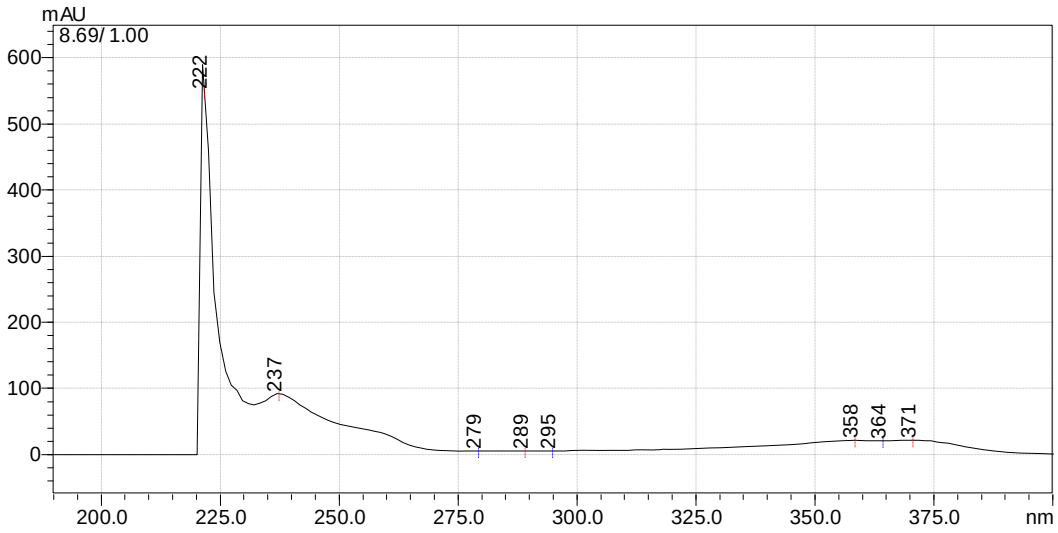


Fig. 21 – UV-spectrum for formulation (60 µg/mL)

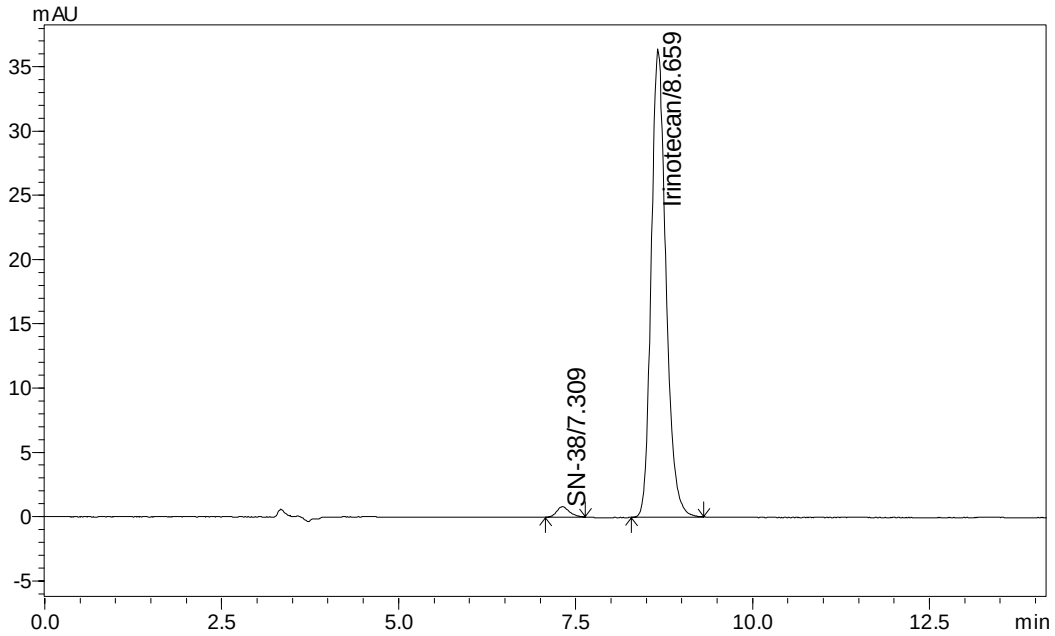


Fig. 22 - HPLC chromatogram for marketed formulation (120 µg/mL)

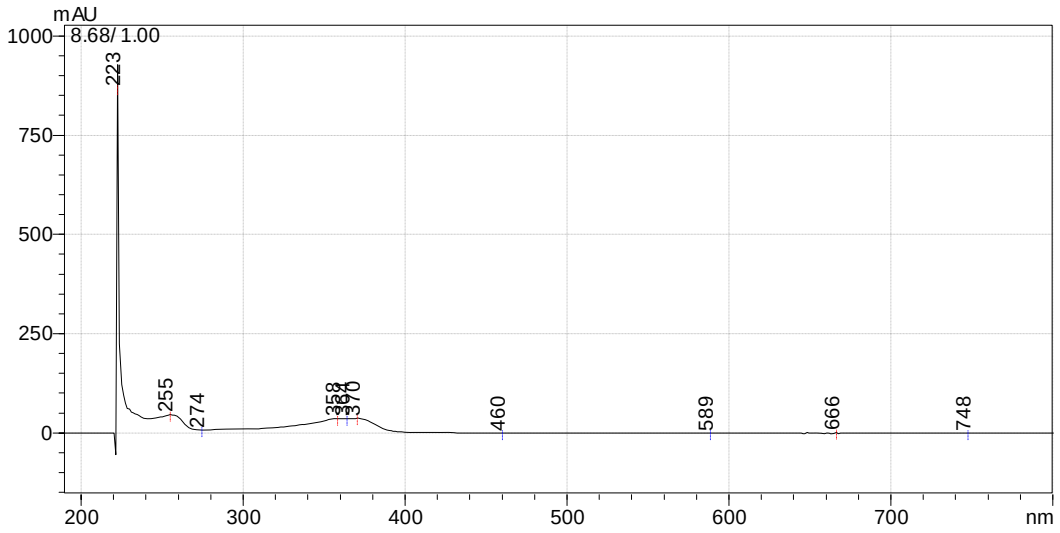


Fig. 23 – UV spectrum for Formulation (120 µg/mL)

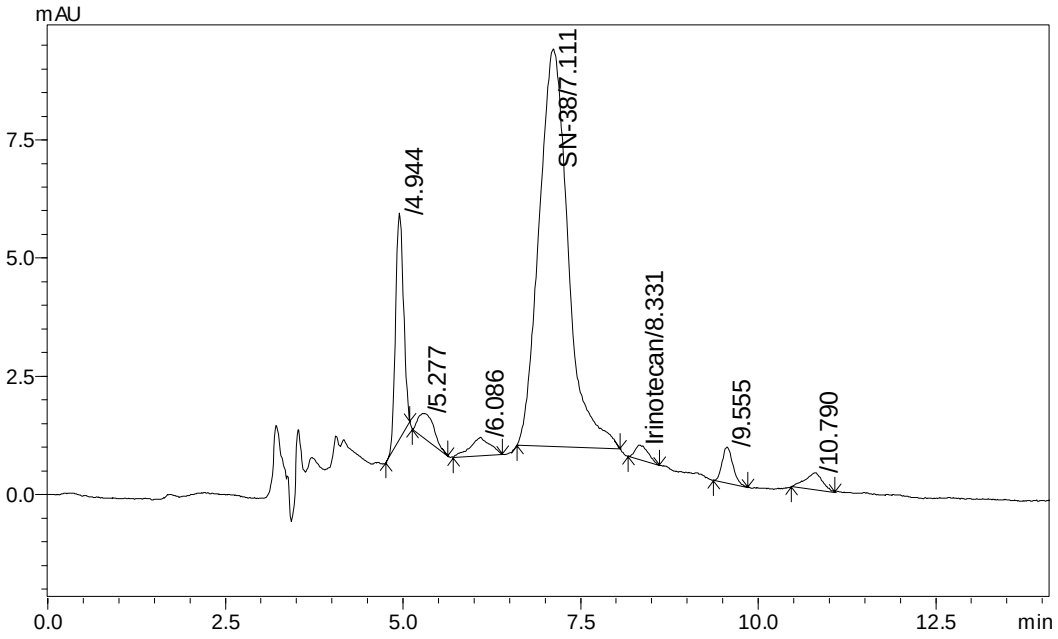


Fig. 24 - HPLC chromatogram of alkaline degradation for standard Irinotecan HCl (20 µg/mL)

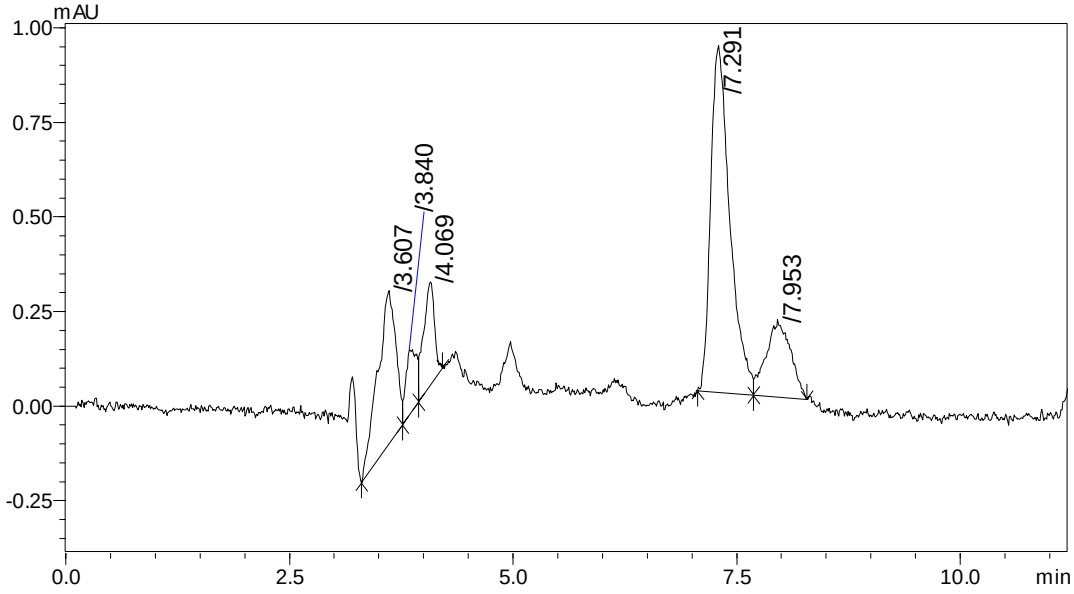


Fig. 25 - HPLC chromatogram of alkaline degradation for test Irinotecan HCl (20 µg/mL)

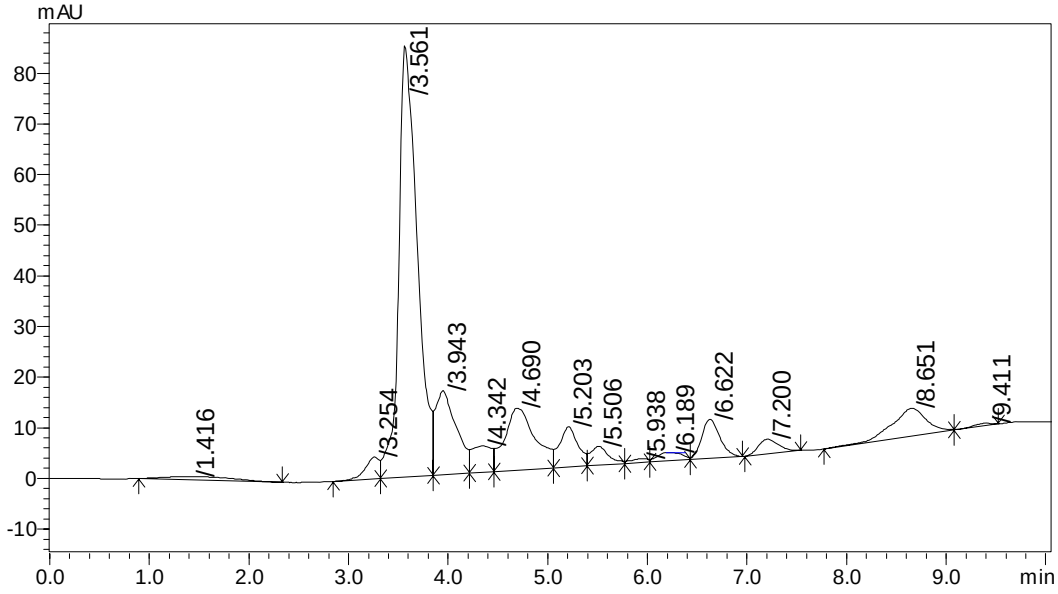


Fig. 26 - HPLC chromatogram of Oxidative degradation for standard Irinotecan HCl (20 µg/mL)

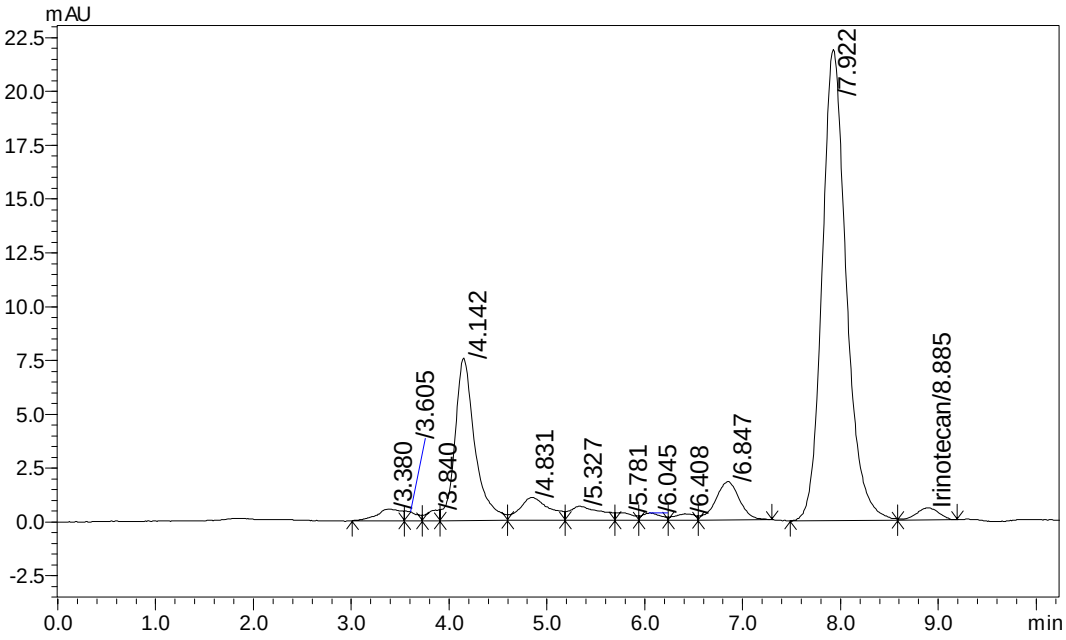


Fig. 27 - HPLC chromatogram of Oxidative degradation for test Irinotecan HCl (20 µg/mL)

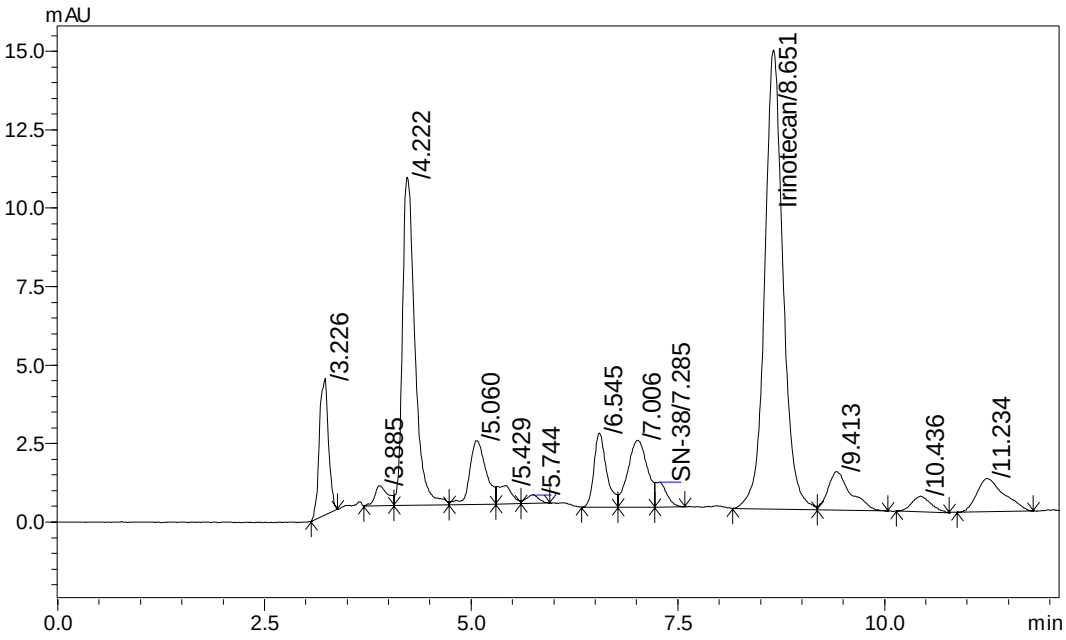


Fig. 28 - HPLC chromatogram of Acid degradation for standard Irinotecan HCl (20 µg/mL)

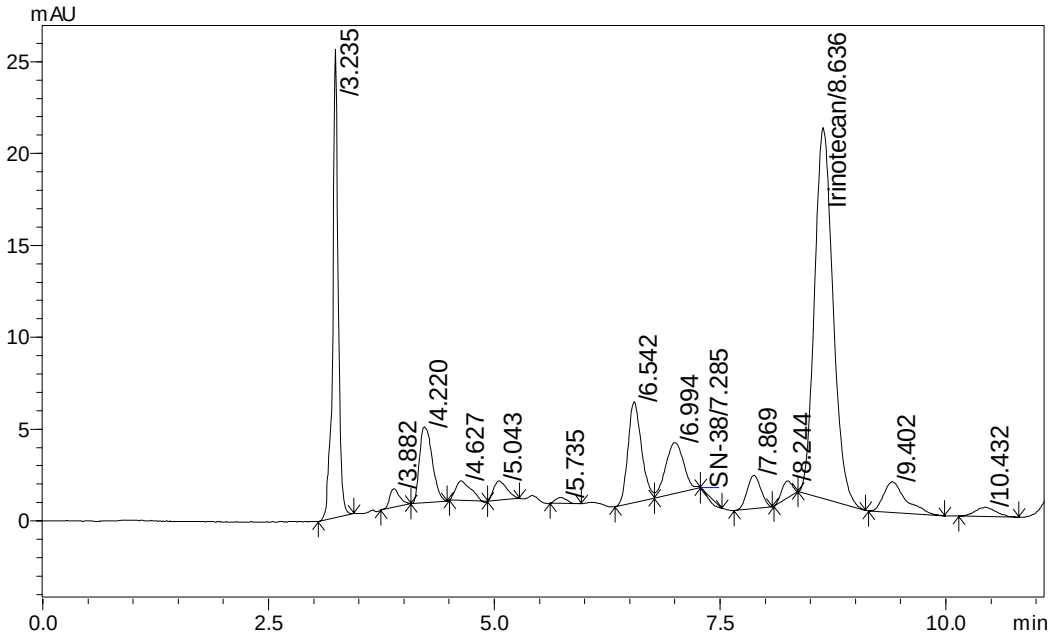


Fig. 29 - HPLC chromatogram of Acid degradation for test Irinotecan HCl (20 µg/mL)

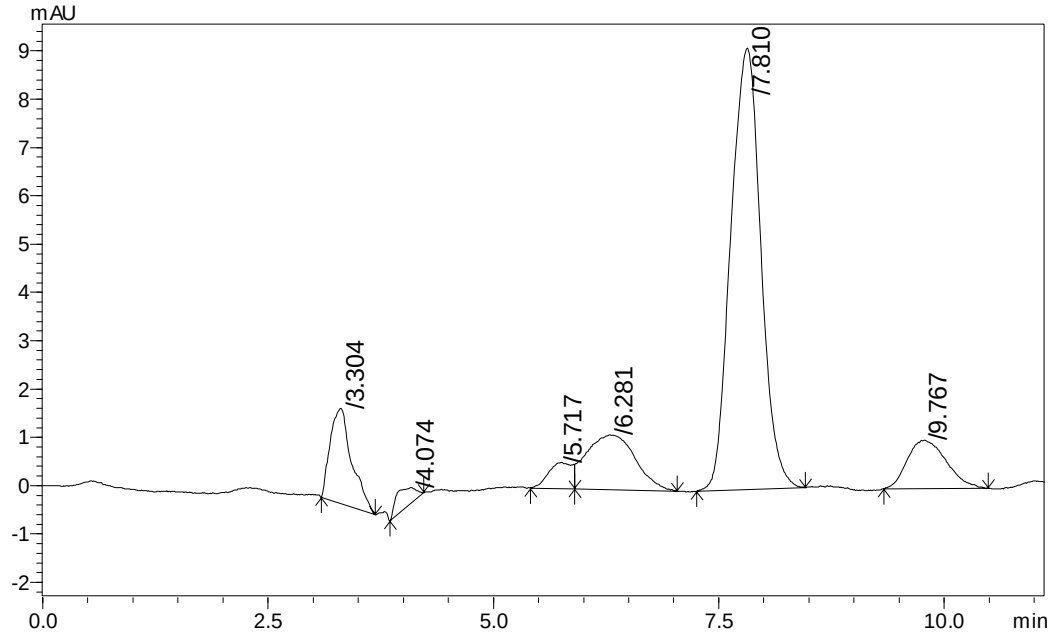


Fig. 30 - HPLC chromatogram of Photolytic degradation for standard Irinotecan HCl (20 µg/mL)

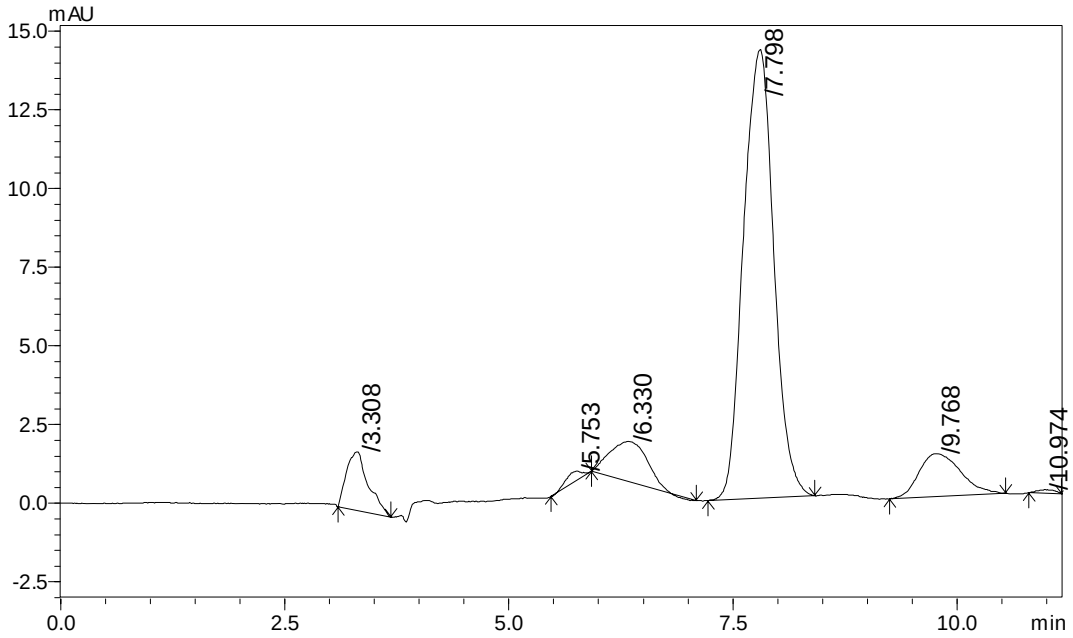


Fig. 31 - HPLC chromatogram of Photolytic degradation for test Irinotecan HCl (20 µg/mL)

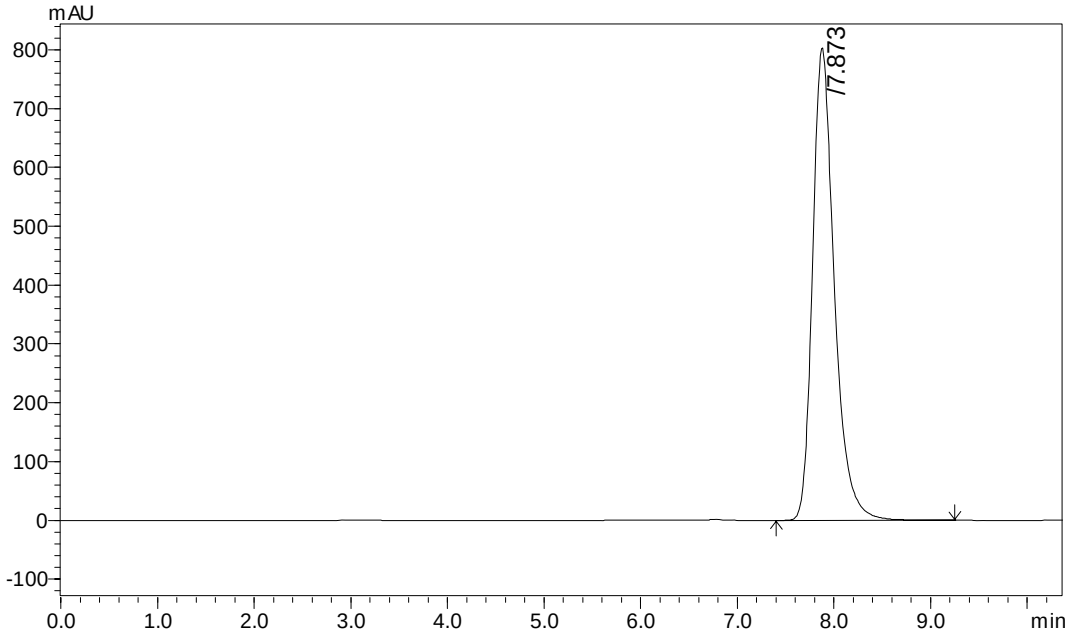


Fig. 32 - HPLC chromatogram of Thermal degradation for standard Irinotecan HCl (20 µg/mL)

CHAPTER -7

RESULTS & DISCUSSION

Chapter 7

RESULTS AND DISCUSSION

HPLC METHOD

A RP-HPLC method was developed and validated for the estimation of Irinotecan HCl and its related substance in injection dosage form by using Phenomenex Luna C_{18} column, 5µ, 250 × 4.60 mm, as a stationary phase and 0.5% trichloro acetic acid: Acetonitrile: Methanol (60: 20: 20 v/v/v) as mobile phase. The flow rate was 1mL/min and the effluents were monitored at 372 nm. The retention time (R_t) for Irinotecan HCl and SN-38 was found to be 8.65 and 7.30.

The percentage of drug in formulation, mean and relative standard deviation were calculated and presented in **Table 8** and the percentage of related substance (SN-38) present in the formulation was also calculated and presented in **Table 9**. The result of analysis showed that the amount of drug present in the formulation is in good correlation with the label claim of the formulation.

VALIDATION OF THE METHOD [42-44]

The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analyzed sample formulations at 50%, 100% and 150% levels. The recovery studies were carried out 6 times of each level and the percentage recovery and percentage relative standard deviation were calculated and given in **Table 10.**

Table 8. Analysis of Formulation for Irinotecan HCl

Drug	Label Claim (mg/mL)	Estimated Amount (mg/mL)	% Label Claim	%RSD*
Irinotecan	20 mg	20.10 mg	100.5%	0.45

*- each value is the mean of six observations

Table 9. Estimation of related substance (SN-38) in Irinotecan HCl formulation

Related substance	Estimated amount (mg/mL)	% Amount present
SN-38	0.0389 mg	0.19%

The percentage recovery of Irinotecan HCl was found to be in the range of 98% and 102% and percentage of related substance (SN-38) was found to be within the limit of 0.2% **.**The recoveries of standard drugs were found to be accurate and within the specified limits.

1. Accuracy, precision and recovery:

Table 10: Accuracy (Recovery studies)

Drug	Label Claim mg/mL	Spike Level (%)	Amount of drug added (mg)	Amount of drug recovered (mg)	Mean Peak area	Percentage Recovery ± SD	%RSD*
Irinotecan	20 mg	50	50	49.27	330386	98.54 ± 0.45	0.45
		100	100	99.10	670722	99.10 ± 0.40	0.40
		150	150	148.93	998654	99.28 ± 0.35	0.36

*-Each value is the mean of six observations.

Acceptance Criteria [42-44]

For an assay method, mean recovery should be 100% ± 2% at each concentration over the range of 50-150% of the target concentration.

From the data obtained, it was observed that the recoveries of standard drugs were found to be accurate and within the specified limits.

Precision:

The precision of the method was determined by studying repeatability and reproducibility. The area of drug peaks and percentage relative standard deviation of intraday and inter day were calculated and presented in **Table 11(a) and 11(b)**. The results revealed that the developed method was found to be reproducible in nature.

Table 11(a). Intraday Studies

No of Injection	Conc. of Irinotecan (µg/mL)	Peak Area	%RSD*
6	60	330193 332491 334384 330569 331726 332549	0.45

*-Each value is the mean of six observations.

Acceptance criteria [42-44]

The % relative standard deviation of peak areas of Irinotecan HCl should not be more than 2.0

From the data obtained, it was observed that the % standard deviation was found to be 0.45 and the value was within the specified limits.

Table 11(b). Inter day studies:

Day	Conc. of Irinotecan (µg/mL)	Peak Area	% RSD*
Day 1	120	522253 524775 526365 522786 524543 525465	0.29
Day 2	120	523153 525675 527765 522786 525543 525465	0.35
Day 3	120	522153 525775 527865 522786 525543 525465	0.40

*-Each value is the mean of six observations.

Acceptance criteria [42-44]

The % relative standard deviation of peak areas of Irinotecan HCl should not be more than 2. Since the values obtained are less than 2%. The acceptance criterion is fulfilled.

2. Repeatability:

Table 12. Repeatability:

Conc. of Irinotecan (µg/mL)	Peak Area	% RSD*
120	522153 525775 527865 522786 525543 525465	0.40

*-Each value is the mean of six observations.

% RSD value for repeatability was found to be (0.40) within the limit of ICH guidelines. Since the method was highly precise.

3. **Linearity:**

Irinotecan HCl and SN-38 were found to be linear in the range of 30 to 150 µg/mL respectively.

The correlation coefficient of Irinotecan HCl and SN-38 were found to be 0.999 and 1.0 respectively. The linearity range of Irinotecan HCl and SN-38S were shown in **Table 13** and **14** respectively. The calibration curves were plotted before peak area and concentration of the standard solutions (**Fig: 33** and **34)**.

The calibration graph showed linear response over the range of 30 to 150 µg/mL concentrations. The range demonstrates that the method is linear outside the limits of expected use.

Table 13. Linearity range of Irinotecan HCl

S.NO.	Conc. of Irinotecan (µg/mL)	Peak areas of Irinotecan
1	30	178350
2	60	335147
3	90	490370
4	120	640000
5	150	791000

Fig. 33 Calibration curve of Irinotecan HCl

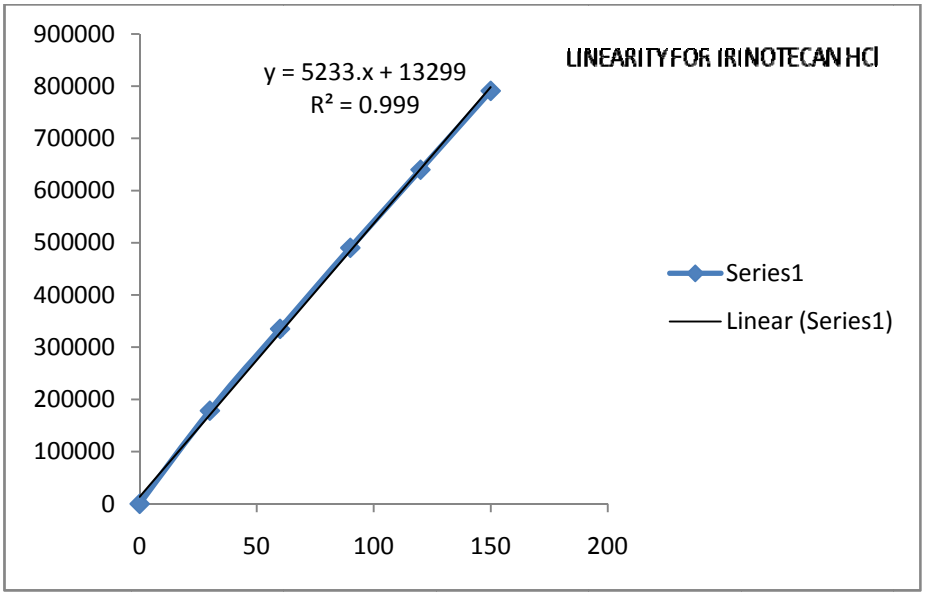
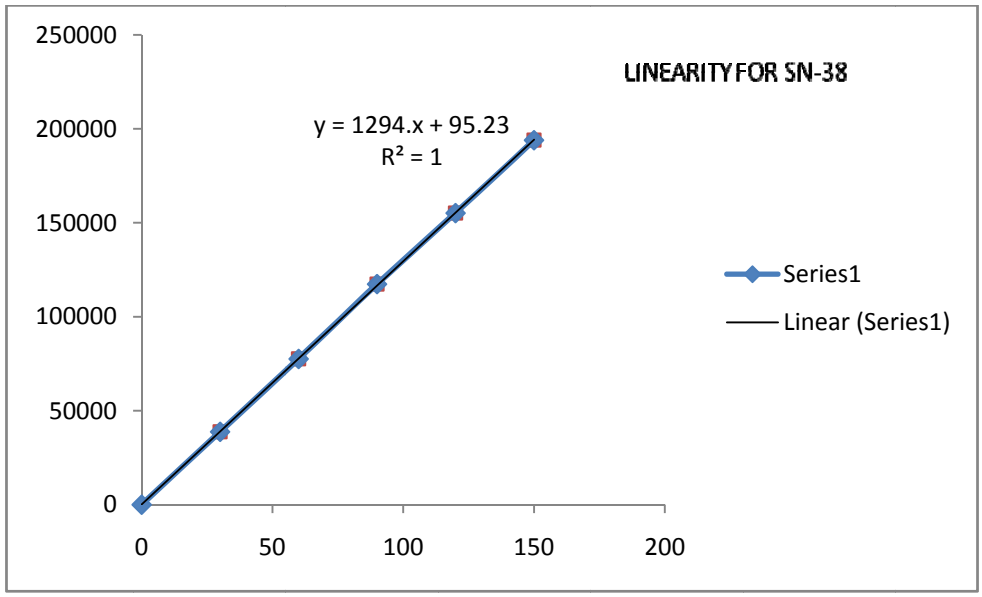


Table 14. Linearity range of SN-38

S.NO.	Conc. of SN-38 (µg/mL)	Peak area of SN-38
1	30	38796
2	60	77592
3	90	117399
4	120	155184
5	150	193980

Fig. 34 Calibration curve of SN-38



Acceptance Criteria [42-44]

Coefficient of correlation should be not less than 0.999; the results of linearity were found to be within the specified limits.

4. Ruggedness:

Table 15. Ruggedness studies

Drug name	Concentration (µg/mL)	Mean peak area	%RSD*
Day-1 analyst-1			
Irinotecan HCl	60	332549	0.450
Day-2 analyst-2			
Irinotecan HCl	60	332495	0.443

*-Each value is the mean of six observations.

The sample was analyzed by two different analyst with same instrument on different days and the results of the analysts were found to be within the limits. Thus the method is rugged.

5. Limit of Detection and Limit of Quantification:

The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3.3). LOD of Irinotecan HCl was found to be 0.014 µg/mL. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Irinotecan HCl was found to be 0.045 µg/mL.

6. Robustness:

The Robustness studies were performed for the standard solutions and were presented in **Table 16.** The assay values were within the limits that the developed method is robust.

Table 16. Robustness Studies:

Chromatographic Condition	Retention Time(R_t)	% Assay
	Irinotecan HCl	*Irinotecan HCl*
Wavelength at 372	8.68 ± 0.05	100.5%
Wavelength at 375	8.59 ± 0.05	97.82%
Column C_{18} (Nucleosil)	7.32 ± 0.07	98.27%
Column C_{18} (Phenomenex)	8.68 ± 0.05	99.12%
Flow rate 1.0 mL/min	8.65 ± 0.06	98.89%
Flow rate 1.2 mL/min	6.67 ± 0.06	95.58%

6. System Suitability:

The system suitability studies were performed for the standard solutions and were presented in **Table 17.** The values obtained demonstrated the suitability of the system for the analysis of the above drug combination.

Table 17. System suitability studies:

Validation Parameters	*Irinotecan HCl*
Linearity range (µg/mL)	30-150
Correlation co-efficient (R^2)	0.999
LOD (µg/mL)	0.014
LOQ (µg/mL)	0.045
Intraday (%RSD)*	0.45
Interday (%RSD)*	0.34
Repeatability (%RSD)*	0.40
Accuracy (%)	98.23-100.50
Peak purity index	1.0000
Resolution factor (Rs)	3.2
No. of theoretical plates (N)	8439.4
Capacity factor (K')	2.125
High equivalent to theoretical plates (HETP)	17.60
Tailing factor	0.91

*-Each value is the mean of six observations.

DEGRADATION STUDIES:

Table 18. Forced degradation studies

Type of degradation	Standard (pure)			Formulation (Test)		
	% drug remained	% degradn to R.S	% degradn Unknown	% drug remained	% degradn to R.S	% degradn Unknown
Acid degradation	41.81%	1.35%	56.83%	45.71%	NIL	54.28%
Alkaline degradation	1.29%	76.98%	21.73%	NIL	77.22%	22.78%
Oxidative degradation	2.63%	3.77%	93.6%	1.51%	NIL	98.49%
Photolytic degradation	NIL	NIL	100%	NIL	NIL	100%
Thermal degradation	NIL	NIL	100%	***	***	***

OBSERVATION:

1. Acid degradation:

***Standard*:** 41.81% drug was remained without degradation, 1.35% drug was degraded as related substance and 56.83% drug was degraded to unknown products.

Test **:** 45.71% drug was remained without degradation and 54.28% drug was degraded to unknown products.

2. Alkaline degradation:

***Standard*:** 1.29% drug was remained without degradation, 76.98% drug was degraded to related substance and 21.73% drug was degraded to unknown products.

Test **:** 77.22% drug was degraded to related substance and 22.78% drug was degraded to unknown products. Here we observed complete degradation.

3. Oxidative degradation:

***Standard*:** 2.63% drug was retained without degradation, 3.77% drug was degraded to related substance and 93.6% drug was degraded to unknown products.

Test **:** 1.51% drug was retained without degradation and 98.49% drug was degraded to unknown products.

Photolytic degradation:

***Standard*:** Complete degradation to unknown products.

Test **:** Complete degradation to unknown products.

1. Thermal degradation:

***Standard*:** Complete degradation to unknown products.

INFERENCE:

- In Acid degradation, there was 40 – 45 % unchanged Irinotecan HCl.
- In Alkaline degradation, maximum degradation was done by converting to related substance.
- In Oxidative, Photolytic and Thermal degradation, there was an almost complete degradation to unknown products.

CHAPTER -8

CONCLUSION

Chapter 8

SUMMARY AND CONCLUSION

SUMMARY:

- A RP-HPLC method was developed to detect and quantify the related substance (SN-38) in marketed Irinotecan HCl formulation, where retention times of both were found to be 8.6 and 7.3 min respectively.
- The proposed method was validated for linearity, range, specificity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines.
- The percentage of related substance (SN-38) in the formulation was found to be within the limits (0.2% max).
- The forced degradation studies are also found to be satisfactory.

CONCLUSION:

It was concluded that the developed RP-HPLC method was found to be very simple, reliable and selective for providing satisfactory accuracy and precision. The methods are suitable for routine quantitative analysis in pharmaceutical dosage forms. A stability indicating assay has been established by following the recommendations of ICH guidelines.

Since the Quantification of related substance (impurity profile) in Qualitative analysis plays a major role in the health benefits, this study will be useful for Quality control of Irinotecan HCl in Pharmaceutical industries.

CHAPTER -9

REFERENCES

Chapter 9

REFERENCES

1. Satinder Ahuja, Karen Mills Alsante; Hand book of isolation and characterization of impurities in pharmaceuticals; volume 5 of separation science and technology, Elsevier, 2005, pp: 2-25.
2. International Conference on Harmonization (2000) Draft Revised Guidance On Impurities In New Drug Substances. Federal Register Q3A(R) 65 (140): 45085.
3. International Conference on Harmonization (2000) Draft Revised Guidance On Impurities In New Drug Products. Federal Register Q3B(R) 65 (139): 44791.
4. International Conference on Harmonization (1997) Impurities, Q3C- Guidelines for Residual Solvents, Q3C. Federal Register 62(247): 67377.
5. International Conference on Harmonization (1999) Specifications, Q6A: Test Procedures and Acceptance Criterial for New Drug Substances and New Drug Products. Chemical substances 65 (146):67488.
6. ICH Q3AR Guideline; Impurity Testing Guideline Impurities in New Drug Substances; ICH Step 5, pp: 77-85.
7. Farmer S, Anderson P, Burns P and Velagaleti R Forced (2002) Degradation of Ibuprofen in Bulk Drugs and Tablets. Pharmaceutical Technology 28: 42.
8. Sanjay B. Bari, Bharati R. Kadam, Yogini S. Jaiswal and Atul A. Shirkhedkar; Impurity profile: Significance in Active Pharmaceutical Ingredient; Eurasian journal of analytical chemistry; Volume 2, number 1, 2007, pp:32-53.
9. Roy J, Islam M, Khan A H, Das S C, Akhteruzzaman M, Deb A K, Alam A H (2001) Diclofenac sodium injection sterilized by autoclave and the occurrence of cyclic reaction producing a small amount of impurity. J Pharma Sci 90: 541
10. Roy J, Mahmud M, Sobhan A, Aktheruzzaman M, Al-Faooque M and Ali E (1994) Marketed vitamin B-complex injectables: stability and mutual interaction. Drug Dev Ind Pharm 20(13): 2157

11. Hoerle S L, Evans K D and Snider B G (1992) HPLC Determination of Impurities in a 3rd Generation Cephalosporine, Eastern Analytical Symposium, November 16-20, Somerset, New Jersey. 12.
12. Roy J, Bhuiyan K, Faraque A, Sobahan M, Al-Farooque M (1997) Injectable ergometrine: stability and packaging for developing countries. Indian Drugs 34(11): 634.
13. Kumar V, Sunder N and Potdar A (1992) Critical factors in developing pharmaceutical formulations-An overview. Part II. Pharma Tech 16:86.
14. Richard J. Smith, Michael L. Webb; Analysis of Drug Impurities; **1**st edn, Blackwell Publishing, 2007, pp: 1-10.
15. Maja J.A. de Jonge, Jaap Verweij; Pharmacokinetic, Metabolic, and Pharmacodynamic Profiles in a Dose-Escalating Study of Irinotecan and Cisplatin; Journal of Clinical Oncology, Vol 18, No 1 (January), 2000: pp 195-203.
16. RS Lloyd, JK Joseph; LG Joseph; Practical HPLC Method Development, John Wiley and Sons, Inc, Noida, 2, 235-251,1997**.**
17. MB David, Validating Chromatographic Methods – A Practical Guide, John Wiley and Sons, Inc, Noida, 6, 1-5,2006.
18. ICH Harmonized Tripartite Guidelines, Validation of Analytical Procedures: Text and Methodology**,** Q2R (1),1994.
19. Center for Drug Evaluation and Research (CDER), 1994. Reviewer Guidance, Validation of Chromatographic Methods.
20. FDA Guidance for Industry. Analytical Procedures and Methods Validation (draft guidance), August 2000.
21. Monika Bakshi and Saranjit Singh. Development of validated stability-indicating assay methods--critical review. J. Pharm. Biomed. Anal. 2002; 28(6):1011-1040.
22. John W. Dolan. Stability-Indicating Assays. LC Troubleshooting. LCGC North America, 2002; 20(4):346-349.
23. Michael J. Smela. Regulatory Considerations for Stability Indicating Analytical Methods in Drug Substance and Drug Product Testing. American Pharmaceutical Review. 2005; 8(3):51-54.

24. Donald D. Hong and Mumtaz Shah, Development and validation of HPLC Stability-indicating Assays, In: Sens T. Carstensen, C.T.Rhodes, editors Drug Stability-Principle & Practice. 3rd Edition. New York: Marcel Dekker Inc. 2008; p. 332.
25. Indian pharmacopoeia 2007; volume: 2, pp: 1243-1244.
26. Drug Bank; Open data drug & Drug Target database; Drug created on June 13, 2005 07:24 / Updated on June 02, 2011 15:50.
27. Authorized USP Pending Standards; The United States Pharmacopeial Convention; Version 1, 2008, pp: 1-5.
28. Shende and Gaud; Validated RP-HPLC Analysis of Irinotecan HCl in the Bulk Material and in Pharmaceutical Formulations; Acta Chromatographica 21; 2009, pp: 71–82.
29. Satyanarayana, Kiran Kumar, Appala Raju, Namratha Rani, and Seshagiri Rao; The Estimation of Irinotecan HCl in Parenterals by RP-HPLC; Asian J. Research Chem.. 2(1); Jan-Mar:2009, pp: 54-56.
30. Venkateswara rao , Murali balaram , Ramakrishna , Sankar ganesh and Balamurali krishna ; Validated Reverse Phase HPLC Method for the Determination of Irinotecan in Pharmaceutical Dosage Forms; E-Journal of Chemistry; Volume: 4, No.1, January 2007, pp: 128-136.
31. Owens, Dodds, Fricke, Hanna, Crews; High-performance liquid chromatographic assay with fluorescence detection for the simultaneous measurement of carboxylate and lactone forms of irinotecan and three metabolites in human plasma. J Chromatogr B Biomed Sci Appl 2003; pp: 66-74.
32. Takahashi, Fujiwara, Sumiyoshi, Isobe, Yamaokab, Yamakido; Salivary drug monitoring of irinotecan and its active metabolite in cancer patients; Cancer Chemother Pharmacol 1997; pp: 40-52.
33. Ali Mohammadi, Farnaz Esmaeili, Rassoul Dinarvand, Fatemeh Atyabi and Roderick b. Walker; simultaneous determination of irinotecan hydrochloride and its related compounds by high performance liquid chromatography using ultraviolet detection; asian journal of chemistry vol. 22, no. 5 (2010), pp: 3966-3972.
34. Mohammed Ishaq, Dr. Vanitha Prakash, Hari Kumar,Usha Rani, Ravi Pratap Pulla; Development and validation of a sensitive reversed-phase HPLC method for the

Determination of irinotecan in bulk and in its pharmaceutical formulation; Journal of Pharmacy Research 2010, 3(10),pp: 2431-2434.

35. Gogineni Ratna Prasad, Srinivas Babu, Suman Avula and Ramana; Development and validation of LC method for the estimation of irinotecan in pharmaceutical dosage form; international journal of research in pharmacy and chemistry 2011, 1(2); pp: 167-170.
36. Saini P. K. ; Jain C. L. ; Singh R. M. ; Mathur S. C. ; Singh G. N.; Determination and validation of irinotecan hydrochloride by RP-UPLC method; The Indian pharmacist 2009; vol. 8, n°83, pp. 55-58.
37. Sushama Talegaonkara, Naseem Akhtara, Roop. K. Khara, Md. Faiyazuddina, Farhan J. Ahmada, Zeenat Iqb la & ManuJaggib; A stability indicating HPTLC method for the analysis of irinotecan in bulk drug and marketed injectables; Journal of Liquid Chromatography & Related Technologies; Volume 34, Issue 14, 2011, pp: 1459-1472.
38. Wei Zhang, Ginger E. Dutschman, Xin Li, Min Ye, Yung-Chi Cheng; Quantitation of Irinotecan and its two major metabolites using a liquid chromatography–electrospray ionization tandem mass spectrometric; Journal of Chromatography B; Volume 877, Issue 27, 1 October 2009, pp: 3038-304.
39. Iman Barilero, Guy G. Chabot Daniel Gandia, Jean-Pierre Armand, Anne Mathieu-Boui ;Simultaneous determination of the camptothecin analogue CPT-11 and its active metabolite SN-38 by high performance liquid chromatography: application to plasma pharmacokinetic studies in cancer patients; Journal of Chromatography, 575 (1992) 275-280.
40. Palle, Venkata raghavendra acharyulu; Process for preparing Irinotecan; pp: 1-11.
41. Laurent P. Rivory, Mark R. Bowles, Jacques Robert and Susan M. Pond; Conversion of Irinotecan (CPT-11) to its active metabolite, 7-Ethyl-10hydroxycamptothecin (SN-38) by human liver carboxyl esterase; Biochemical Pharmacology, Vol.52, pp.1103-1111, 1996.
42. ICH Harmonized Tripartite Guideline, ICH Q2A, Text on validation of analytical procedures, Mar.1995.
43. ICH Harmonized Tripartite Guideline, ICH Q2B, Validation of analytical procedures: methodology, May.1997.
44. ICH Harmonized Tripartite Guideline, ICH Q2B (R), Impurities in new drug products, Oct.1999.

CHAPTER -10

GLOSSARY

Chapter 10

GLOSSARY

1. **Degassing:** The process of removing dissolved gas from the liquid mobile phase before or during use. Degassing is done by heating, by vacuum, or by helium purging.
2. **Drift:** The change in the baseline value over time, expressed line mathematically as the slope of the least line squares line fitted to the base line in a specified region chromatogram.
3. **Eluent:** Mobile phase used to perform a chromatographic separation. The liquid that exists through a chromatographic column during a separation.
4. **Equilibration:** The process of bringing a chromatographic solvent (solvent, column and interactive surfaces) to a thermally and chemically stable, usually indicated by a drift_ free base line.
5. **External standard:** A separate sample that contains known quantities of the same compounds that are in unknown sample. External standards are used for quantitation by matching the detector response of a component peak to a point on a calibration curve for that component. The calibration curve is generated from a separately processed standard (or a set of standards).
6. **Fronting peak:** An asymmetrically shaped chromatographic peak in which the front part of the peak (before the apex) contains more area than the rear of the peak. The asymmetry factor for fronting peak has valve less than one. The opposite of fronting peak is a tailing peak.
7. **Gradient elution:** Also called as solvent programming, a technique for decreasing the separation time by increasing the mobile phase strength over time during a chromatographic separation. Gradients can be continuous or stepwise. Binary (2-solvent), ternary (3-solvent) and quaternary (4- solvent) gradients are used routinely in HPLC.
8. **Integration:** The mathematically process of calculating an area such as a chromatographic peak that is bounded in a part or in a whole by a curved line.

9. **Isocratic:** The condition in which the solvent composition, flow rate and the temperature are constant during a chromatographic run, the condition in which the solvent composition is constant during a chromatographic run.
10. **Linearity:** The condition in which detector response is directly proportional to the concentration or amount of a component over a specified range of component concentration or amounts. A calibration curve is a straight line when the standard concentration is within the linear response range of the detector. The chromatography, accurate quantitation requires linearity of the detector response over the range of actual sample concentration or amounts.
11. **Mobile phase:** The fluid (gas or liquid) that carries solutes through a chromatographic column. In LC the liquid that is pumped through the fluid path of the chromatographic system and into which the samples are injected.
12. **Plate count:** A measure of the observed chromatographic resolution based on its equivalency to the number of the theoretical plates that would provide the same resolution.
13. **Resolution:** The extent to which a chromatographic column separates components from each other. Mathematically defined, resolution is the difference between the peak retention time of a selected peak and the peak preceding it's multiplied by a constant of 1.178, and then divided by the sum of the peak widths at 50% of peak height. It is used to monitor the separation of eluting components and to establish system efficiency.
14. **Retention time:** The time that elapses between the injection of a sample and the appearance of the peak maximum of a component in a sample.
15. **R_t ratio:** The retention time of a component divided by the retention time of its reference peak.
16. **System suitability**: An application that applies a set of standard criteria to test if an entire chromatographic system and the methodology are working within acceptable limits. Empower software bases the system suitability testes on standard laboratory calculations, including United States pharmacopoeia (USP) guidelines and calculations. Empower software procedures reports showing statistical accuracy and reproducibility of the chromatographic system data.

17. **Tailing factor:** A measure of peak symmetry where a symmetrical peak has a tailing factor of 1. As tailing increases peak symmetry increases for system suitability the tailing factor is the width of the peak at 5% height divided by two times the distance from the peak maximum to the leading edge of the peak (where the distance is measured at point 5% of the peak height from the baseline).
18. **Capacity factor:** A chromatographic parameter that measures retention time of a sample molecule relative to the column dead volume.
19. **Acceptance criteria:** Numerical limits ranges or other suitable for acceptance of the results of analytical procedures.
20. **Detection limit:** The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value.
21. **Drug product:** A finished dosage form, for example a capable tablet or solution that contains a drug substance, but not necessarily in association with one or more other ingredients.
22. **Drug substance:** Active ingredient: an active ingredient that is intended to furnish pharmacological activity or other direct effect the structure or any function of the human body. The active ingredient does not include intermediates used in the synthesis of such ingredient. The term includes those components that may undergo chemical change in the manufacture of the drug product and be present in the drug product in a modified form intended to furnish the specified activity or effect.
23. **Reagent:** For analytical procedures any substance used in a reaction for the purpose of detecting measuring examining or analyzing other substance.
24. **Specification:** The quality standards (i.e.., tests, analytical procedures, and acceptance criteria) provided in an approved application to confirm the quality of the drug substances, drug products, intermediates, raw materials, reagents, and other components including container closure systems, and in-process materials.
25. **Spiking:** The addition of small known amount of a known compound to a standard, sample, or placebo, typically for the purpose of confirming the performance of an analytical procedure of an analytical procedure or the calibration of an instrument.

26. **Working standards:** A standard that is qualified against and used instead of the reference standard (also known as in-house or secondary standards).
27. **Response factor:** the response of drug substance or related substances per unit weight.

    Response factor=response (in response units)/concentration (in mg/mL)

28. **Relative response factor:** the ratio of the response factor of individual related substance to that of a drug substance to correct for differences in the response of related substances and that of the drug substance.

    Relative response factor = response factor of individual related substance/response factor of drug substance.